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#### Document Version

Final published version

#### Citation (APA)

Fecker, T. (2026). *Opportunities and Challenges in Applying Defined Co-cultures for Microbial Biotechnology*. [Dissertation (TU Delft), Delft University of Technology]. <https://doi.org/10.4233/uuid:17abef0b-c4b1-4715-91de-94c10004c73c>

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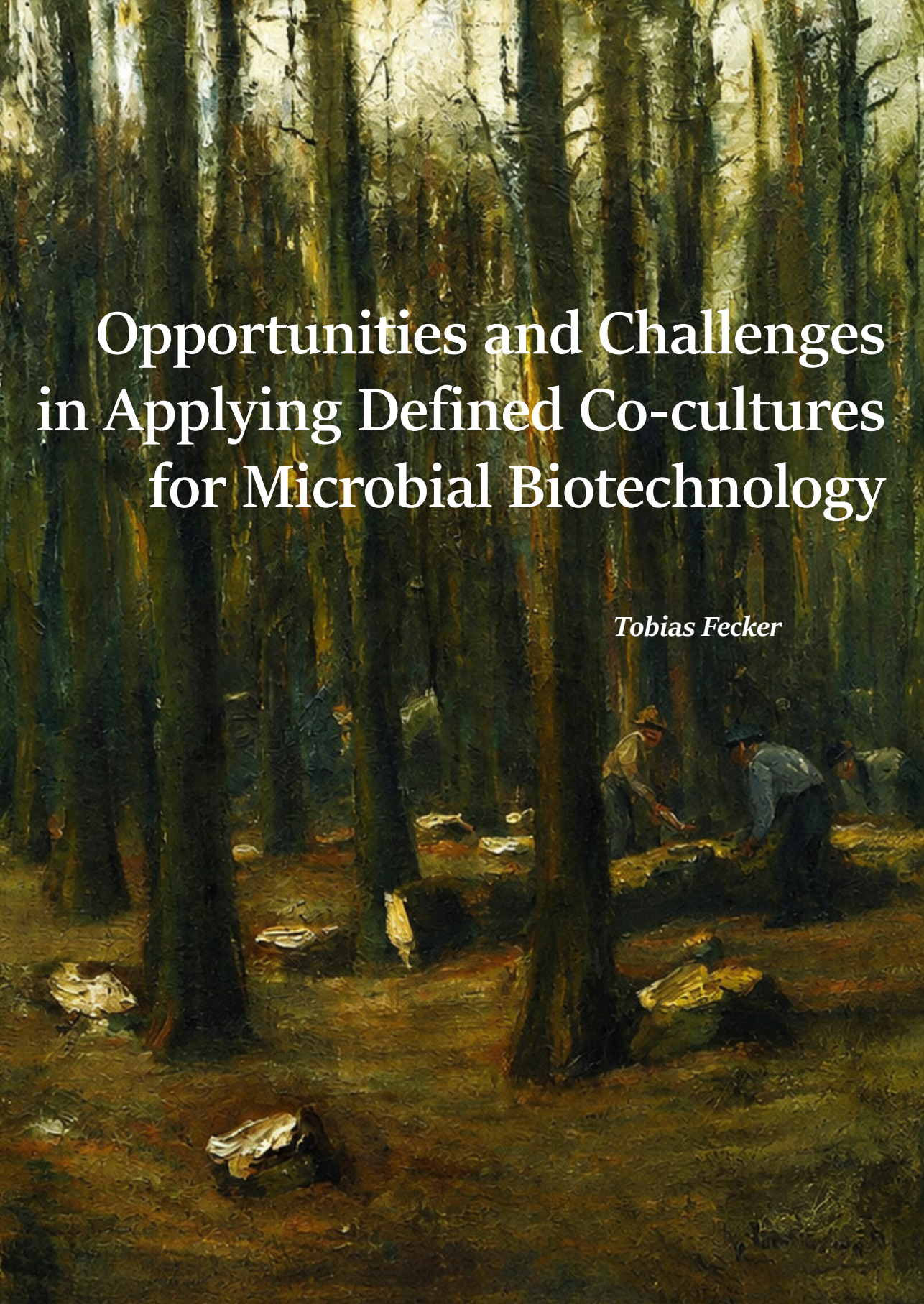
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The background of the cover is a painting of a forest. Tall, slender trees with light-colored bark stand vertically, creating a sense of height and depth. The ground is covered in brown earth and fallen logs. In the lower right, three people are visible, engaged in what appears to be logging or forestry work. One person is standing and looking down, while two others are bent over, working on a log. The overall tone is somber and naturalistic, with a focus on the textures of the trees and the ground.

# Opportunities and Challenges in Applying Defined Co-cultures for Microbial Biotechnology

*Tobias Fecker*



# **Opportunities and Challenges in Applying Defined Co-cultures for Microbial Biotechnology**

Dissertation

For the purpose of obtaining the degree of doctor  
at Delft University of Technology  
by the authority of the Rector Magnificus Prof.dr.ir. H. Bijl,  
chair of the Board for Doctorates  
to be defended on 29 October 2026 at 12:30

by  
**Tobias FECKER**

This dissertation has been approved by the promotor.

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The research presented in this thesis was performed at the Industrial Microbiology Section, Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, the Netherlands. This project was funded by Delft University of Technology.

|             |                   |
|-------------|-------------------|
| Layout:     | T. Fecker         |
| Printed by: | Ridderprint bv    |
| ISBN:       | 978-94-6518-461-6 |

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Wer will was Lebendiges erkennen und beschreiben,

Sucht erst den Geist herauszutreiben,

Dann hat er die Teile in seiner Hand,

Fehlt, leider! nur das geistige Band.

Who would describe and study something living

First drives out the spirit from it;

Then the parts are in his hand,

But the living bond is gone.

Goethe, Faust. Der Tragödie erster Teil, 1808. Studierzimmer,  
Mephistopheles zum Schüler

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# Summary

The natural world is shaped by ecological networks. In all geographical contexts, species coexist and interact with other species, in often complex synergistic or antagonistic ways. This interplay is also apparent at the microbial scale. In the human gut microbiome, for example, dozens of species carve out any available niche to access nutrients, allowing them to persist. To do this, they can form mutualistic alliances with other species, feed on their metabolic products or exploit alliances between other species by intercepting their exchanged nutrients. Such complex networks confer stability by reducing the possibility of opportunistic species taking over, enable formation of new products, and make near-complete use of all available nutrients. Microbial co-cultures exhibiting these advantages are ubiquitous in food fermentation processes, where they have for millennia been used to ferment bread, beer, tea leaves, and milk. In modern food fermentation processes, co-cultures often comprise a defined selection of species and strains, optimized for the intended application.

Industrial biotechnology, which uses microbial strains to convert carbon sources into economically viable products, has been at the forefront of the bioeconomy due to its potential to replace current fossil-based processes by more sustainable bioprocesses. However, many current bioprocesses, including the large-scale production of bioethanol from corn starch, rely on single, optimized strains of microbes. The processes face challenges since they require strict control of their environment to produce optimally and often depend on single carbon substrates such as glucose or sucrose. Additionally, they are limited by their ability to produce certain products, due to difficulties of integrating complex pathways into the host strain's metabolism.

The overall theme of this thesis is to explore techniques and potential applications of co-cultures in microbial biotechnology. To do this, potential advantages for co-cultures over monocultures are reviewed, strategies to facilitate operation of co-cultures are proposed, relevant ecological dynamics explored and practical insights into high-throughput screening systems are provided that can be applied for co-culture design.

**Chapter 1** discusses the potential of defined co-cultures to offer solutions to

challenges faced in monoculture-based industrial biotechnology. Applications are divided into two categories based on whether formation of their products yields ATP: dissimilatory and assimilatory products. Dissimilatory products, whose production from the carbon source provides the cell with ATP, include high-volume products like bioethanol. Due to their large volume, incremental yield or productivity increases can confer high economic benefits. The chapter outlines how, by using defined co-cultures in these processes, byproducts can be sequestered, increasing the product yield. Also, in mixtures of different substrates, using co-cultures of specialist strains can prevent a gradual loss of the ability to break down certain sugars observed in monocultures of ‘generalist’ strains able to consume all sugars. For assimilatory products, including plant metabolites, dyes, or fragrances, co-cultures offer potential solutions to challenges related to the engineering of complex heterologous pathways, host-enzyme incompatibility, and intermediate build-up. While promising, co-cultures also harbor novel challenges. Negative interactions between the co-culture members can arise and the relative abundance of strains must be maintained at an optimal level throughout the industrial fermentation process.

One challenge of designing co-cultures of specialist *Saccharomyces cerevisiae* strains to consume substrate mixtures is related to imbalances in the strains’ growth kinetics. While the co-cultures offer efficiency advantages over generalist strains that consume the available sugars sequentially rather than concomitantly, their overall conversion time is dictated by the slowest fermenter. When considering conversion of lignocellulosic hydrolysates, this is often a pentose-fermenting strain. **Chapter 2** proposes a strategy to overcome this bottleneck in sequential batch fermentation. In sequential batch processes, the growth medium is periodically replaced after substrate depletion. When performed in bioreactors, sequential batch cultivation can be programmed to incorporate a biomass settling phase. By inactivating *ACE2* in a slow-growing, diploid, arabinose-consuming *S. cerevisiae* specialist strain, it was engineered to form multicellular clusters that sediment rapidly. This enabled a selective increase in biomass concentration of this strain during the settling phases in sequential batch fermentation and improved kinetics in the subsequent batch cycles. The strategy reduced the overall conversion time by 30% in monocultures and enabled near-simultaneous consumption of arabinose and glucose in co-cultures with a glucose-specialist strain. This study demonstrates how engineering a multicellular phenotype can balance growth kinetics of co-cultures in prolonged sequential batch fermentations.

Defined co-cultures can be designed to harbor mutualistic interactions, which improves stability and prevents the extinction of individual strains. However, these interactions often promote growth of non-productive cheater strains that exploit the mutualistic exchange of nutrients. In spatially defined conditions, e.g., on agar plates,

such cheater populations can be detrimental for the overall culture growth if they blockade nutrient exchange of the cooperators. **Chapter 3** describes a combination of theoretical and experimental approaches to explore the factors underlying the success of cooperators in these environments. Surprisingly, a single parameter - the time of first contact – was shown to determine whether the co-culture could proliferate. This simple model is able to capture the governing dynamics of this complex system and provides important insights into understanding real-world cooperator-cheater dynamics. These insights may be translated into biotechnological applications that depend on mutualistic interactions, such as fermentative production of yoghurt or kombucha.

As an alternative to the rational design of co-cultures, microdroplets can be employed as a high-throughput screening tool. By encapsulating single cells or co-cultures into picolitre-sized microdroplets, researchers can screen libraries of up to 1010 candidates per mL volume without the risk of the fastest-growing strains outcompeting slower, more productive strains. However, since the droplets are in close proximity to one another, the approach depends on the strict compartmentalization of all available metabolites, such that they cannot transfer to neighboring droplets and interfere with other cells. **Chapter 4** describes that such isolation cannot be guaranteed for some biologically relevant metabolites, such as ethanol or weak acids. Especially alcohols, due to their amphiphilic character, transfer freely among droplets, which can impact yield calculations for cells able to consume these metabolites. Other metabolites with high hydrophilicity, such as sugars, are more likely to remain inside the droplets. The octanol-water partition coefficient  $\log P$  is identified as a physical parameter to predict potential transfer risk. This study therefore establishes guidelines for the choice of substrates and products to ensure transfer-free screening setups.

In summary, this thesis moves from the theoretical potential of microbial co-cultures to their application in microbial biotechnology. By combining genetic engineering, ecological modeling, and screening tool development, practical strategies for design and process integration of these cultures are presented. These advances may help pave the way for the more widespread adoption of microbial co-cultures in biotechnological applications.

# Samenvatting

Translation provided by Gabriela van Leersum

De natuurlijke wereld wordt gevormd door ecologische netwerken. In elke geografische context leven soorten naast elkaar en staan ze in wisselwerking met andere soorten, vaak op complexe synergetische of antagonistische manieren. Deze wisselwerkingen zijn ook zichtbaar op microbiële schaal. Dit geldt bijvoorbeeld voor het menselijke darmmicrobioom, waarin tientallen soorten elke beschikbare niche veroveren om daarmee toegang te krijgen tot voedingsstoffen om te overleven. Teneinde dit te doen, kunnen soorten coöperatieve allianties aangaan met andere soorten, zich voeden met stofwisselingsproducten van andere soorten, of allianties tussen andere soorten uitbuiten door hun uitgewisselde voedingsstoffen te onderscheppen. Dergelijke complexe netwerken zorgen voor stabiliteit door de kans te verkleinen dat opportunistische soorten de overhand krijgen, ze maken de vorming van nieuwe producten mogelijk en benutten nagenoeg alle beschikbare voedingsstoffen. Microbiële co-culturen die deze voordelen vertonen, zijn alomtegenwoordig in voedsel fermentatieprocessen, waar ze al duizenden jaren worden gebruikt voor productie van brood, bier, thee en gefermenteerde zuivelproducten. In moderne voedsel fermentatieprocessen bestaan co-culturen vaak uit een gedefinieerde selectie van soorten en stammen, geoptimaliseerd voor de beoogde toepassing.

Industriële biotechnologie gebruikt microbiële stammen om koolstofbronnen om te zetten in economisch haalbare producten en speelt een sleutelrol in de bio-economie vanwege haar potentie om huidige, op fossiele grondstoffen gebaseerde processen te vervangen door duurzamere bioprocessen. Veel huidige bioprocessen, waaronder de grootschalige productie van bio-ethanol uit maïszetmeel, zijn afhankelijk van enkele, geoptimaliseerde microbiële stammen. Deze processen brengen uitdagingen met zich mee omdat ze een strikte controle van de procescondities vereisen om optimaal de gewenste producten te produceren en vaak afhankelijk zijn van één enkele grondstof, zoals glucose of sacharose. Bovendien worden ze beperkt door hun vermogen om bepaalde producten te produceren, vanwege de moeilijkheden bij het integreren van complexe routes in de stofwisseling van de gastheerstam.

Het overkoepelende thema van dit proefschrift is het verkennen van technieken en potentiële toepassingen van co-culturen in de microbiële biotechnologie. Hiertoe worden, op basis van literatuuronderzoek de potentiële voordelen van co-culturen ten opzichte van monoculturen geïdentificeerd, worden strategieën voorgesteld om de werking van co-culturen te vergemakkelijken, wordt de relevante ecologische dynamiek onderzocht en worden praktische inzichten verschaft in high-throughput screeningsystemen die kunnen worden toegepast voor het ontwerp van co-culturen.

**Hoofdstuk 1** bespreekt het potentieel van gedefinieerde co-culturen om oplossingen te bieden voor uitdagingen waarmee de op monoculturen gebaseerde industriële biotechnologie te maken heeft. Toepassingen worden onderverdeeld in twee categorieën, afhankelijk van of hun producten ATP opleveren: dissimilatoire en assimilatoire producten. Dissimilatoire producten, waarvan de productie uit de koolstofbron de cel van ATP voorziet, omvatten producten die in grote hoeveelheden worden geproduceerd, zoals bio-ethanol. Vanwege hun grote volume kunnen marginale verhogingen van de opbrengst of productiviteit aanzienlijke economische voordelen opleveren. Het hoofdstuk schetst hoe, door gebruik te maken van gedefinieerde co-culturen in deze processen, bijproducten kunnen worden heropgenomen, waardoor de productopbrengst toeneemt. Ook kan, bij omzetting van mengsels van verschillende substraten, het gebruik van co-culturen van gespecialiseerde stammen een geleidelijk verlies van het vermogen om bepaalde suikers af te breken voorkomen, zoals waargenomen in monoculturen van 'generalistische' stammen die alle suikers kunnen consumeren. Voor assimilatieproducten, waaronder plantenmetabolieten, kleurstoffen of geurstoffen, bieden co-culturen potentiële oplossingen voor uitdagingen die gerelateerd zijn aan het ontwerpen van complexe heterologe productvormingsroutes, incompatibiliteit tussen gastheer en specifieke enzymen, en de ophoping van tussenproducten. Hoewel co-culturen veelbelovend zijn, brengen ze ook nieuwe uitdagingen met zich mee: er kunnen negatieve interacties tussen de leden van de co-cultuur ontstaan en de relatieve cel aantallen van de stammen moet op een optimaal niveau worden gehouden.

Een disbalans in de groeikinetiek van verschillende stammen vormt een uitdaging bij het ontwerpen van co-culturen van gespecialiseerde *Saccharomyces cerevisiae*-stammen voor de consumptie van suikermengsels zoals die voorkomen in hydrolysaten van lignocellulose. Hoewel zulke co-culturen efficiëntievoordelen bieden ten opzichte van generalistische stammen die de beschikbare suikers achtereenvolgens in plaats van gelijktijdig consumeren, wordt hun totale omzettingstijd bepaald door de traagste stam (in de praktijk meestal een pentose-fermenterende stam). **Hoofdstuk 2** stelt een strategie voor om deze bottleneck in langdurige, sequentiële batchfermentaties te overwinnen. In zulke kweeksystemen wordt het kweekmedium na uitputting van het substraat vervangen voor vers medium. Wanneer deze processen worden

uitgevoerd in bioreactoren is het mogelijk om een geprogrammeerde biomassa-bezinkfase op te nemen. Door *ACE2* te inactiveren in een langzaam groeiende, diploïde, arabinose-consumerende *S. cerevisiae*-specialistenstam, werd een stam verkregen die multicellulaire, snel bezinkende celclusters vormde. Dit maakte een selectieve toename van de biomassaconcentratie van deze stam mogelijk tijdens de bezinkfasen in sequentiële batchfermentatie en verbeterde de kinetiek in de daaropvolgende batchcycli. Deze strategie verminderde de totale conversietijd met 30% in monoculturen en maakte een bijna gelijktijdige consumptie van arabinose en glucose mogelijk in co-culturen met een glucose-specialistische stam. Deze studie toont aan hoe het ontwikkelen van een multicellulair fenotype de groeikinetiek van co-culturen in langdurige sequentiële batchfermentaties in evenwicht kan brengen.

Gedefinieerde co-culturen kunnen zo worden ontworpen dat ze coöperatieve interacties door uitwisseling van voedingsstoffen bevorderen, wat de stabiliteit van de cultuur verbetert en het uitsterven van individuele stammen voorkomt. Deze interacties stimuleren echter vaak de groei van niet-coöperatieve 'cheater'-stammen die profiteren van deze uitwisseling. Onder ruimtelijk afgebakende omstandigheden, bijvoorbeeld op agarplaten, kunnen dergelijke 'cheater'-populaties schadelijk zijn voor de algehele groei van de cultuur als ze interfereren met de uitwisseling van voedingsstoffen tussen de samenwerkende stammen. **Hoofdstuk 3** beschrijft een combinatie van theoretische en experimentele benaderingen om de factoren te onderzoeken die ten grondslag liggen aan het succes van coöpererende stammen in deze omgevingen. Verrassend genoeg bleek één enkele parameter – het tijdstip van het eerste contact – te bepalen of de co-cultuur zich kon vermenigvuldigen. Dit eenvoudige model is in staat de bepalende dynamiek van dit complexe systeem weer te geven en biedt belangrijke inzichten in het begrijpen van de 'real-world' coöperator-cheater dynamiek. Deze inzichten kunnen worden vertaald naar biotechnologische toepassingen die afhankelijk zijn van complexe, ecologische interacties, zoals yoghurt- of kombuchaconsortia.

Als alternatief voor het rationeel ontwerpen van co-culturen kunnen microdruppels worden ingezet als high-throughput screeninginstrument. Door afzonderlijke cellen of co-culturen in microdruppels ter grootte van een picoliter in te kapselen, kunnen onderzoekers bibliotheken van tot wel  $10^{10}$  kandidaten per milliliter volume screenen zonder het risico dat de snelst groeiende stammen de langzamere, productievere stammen verdringen. Aangezien de microdruppels zich dicht bij elkaar bevinden, is deze aanpak afhankelijk van de volledige compartimentering van alle beschikbare metabolieten, zodat deze niet kunnen overgaan naar naburige druppels en daarmee andere cellen beïnvloeden. **Hoofdstuk 4** beschrijft dat een dergelijke strikte compartimentering niet kan worden gegarandeerd voor sommige biologisch relevante metabolieten, zoals ethanol of zwakke zuren. Vooral alcoholen worden door hun amfifiele karakter vrij tussen de microdruppels overgedragen, wat

van invloed kan zijn op opbrengstberekeningen voor cellen die deze metabolieten kunnen consumeren. Andere metabolieten met een hoge wateroplosbaarheid, zoals suikers, blijven wel binnen de microdruppels aanwezig. De octanol-waterverdelingscoëfficiënt  $\log P$  werd geïdentificeerd als een fysische parameter om het potentiële overdrachtsrisico te voorspellen. Deze studie stelt daarom richtlijnen vast voor de keuze van substraten en producten om overdrachtstvrije screeningopstellingen te garanderen.

Samenvattend beweegt dit proefschrift zich van het theoretische potentieel van microbiële co-culturen naar de toepassing ervan in de microbiële biotechnologie. Door genetische modificatie, ecologische modellering en de ontwikkeling van screeningmethoden te combineren, worden praktische strategieën voor het ontwerp en de procesintegratie van deze culturen gepresenteerd. Deze vorderingen kunnen helpen om de weg vrij te maken voor een bredere toepassing van microbiële co-culturen in biotechnologische toepassingen.

# Zusammenfassung

Die Natur ist geprägt von ökologischen Netzwerken. Arten koexistieren in unterschiedlichsten geografischen Kontexten und interagieren dabei auf komplexe, teils symbiotische, teils antagonistische Weise miteinander. Dieses Wechselspiel findet auch auf mikrobieller Ebene statt. Im menschlichen Darmmikrobiom beispielsweise besiedeln Dutzende von Arten jede verfügbare Nische, um an Nährstoffe zu gelangen, und sichern so ihr Überleben. Manche Mikroorganismen bilden dafür symbiotische Allianzen mit anderen Arten und ernähren sich von deren Stoffwechselprodukten. Andere nutzen von Dritten gebildete Allianzen aus, indem sie ausgetauschte Nährstoffe abfangen. Solche Netzwerke erhöhen die Stabilität, indem sie die Möglichkeit einer Übernahme durch opportunistische Arten erschweren, und ermöglichen zugleich die Bildung neuer Produkte. Zudem können sie durch eine effektive Nischenausnutzung nahezu alle verfügbaren Moleküle verstoffwechseln. Mikrobielle Co-Kulturen, Mischungen verschiedener Mikroorganismen, mit diesen Vorteilen sind in Lebensmittel fermentationsprozessen allgegenwärtig, wo sie seit Jahrtausenden zur Fermentierung von Brot, Bier, Teeblättern oder Milchprodukten eingesetzt werden. In modernen Lebensmittel fermentationsprozessen sind die Zusammensetzungen der Kulturen genau definiert und für die beabsichtigte Anwendung optimiert.

Die industrielle Biotechnologie nutzt mikrobielle Stämme, um Kohlenstoffquellen in wirtschaftlich relevante Produkte umzuwandeln und spielt damit eine zentrale Rolle in der Bioökonomie. Diese biologisch angetriebene Herstellung relevanter Stoffwechselprodukte hat das Potenzial, fossile Herstellungsprozesse zu ersetzen. Bislang basieren viele aktuelle Bioprozesse, darunter die Herstellung von Bioethanol aus Maisstärke, jedoch auf einzelnen, optimierten Mikrobenstämmen. Diese Verfahren stehen vor Herausforderungen, da sie für optimale Produktion eine streng kontrollierte Umgebung erfordern und oft von einzelnen Substraten wie Glukose oder Stärke abhängig sind. Zudem gibt es bei der Herstellung Einschränkungen im Produktprofil, weil die realisierbare Komplexität der in den Mikroorganismus integrierten Stoffwechselwege begrenzt ist.

Das übergeordnete Thema dieser Arbeit ist die Erforschung von Techniken und

potenziellen Anwendungen von Co-Kulturen in der mikrobiellen Biotechnologie. Zu diesem Zweck werden potenzielle Vorteile von Co-Kulturen gegenüber Monokulturen herausgestellt, Strategien zur Vereinfachung des Betriebs von Co-Kulturen vorgeschlagen, relevante ökologische Dynamiken untersucht und praktische Einblicke in Screening-Systeme gegeben, die für die Entwicklung von Co-Kulturen genutzt werden können.

**Kapitel 1** befasst sich mit den technischen Herausforderungen der auf Monokulturen basierenden industriellen Biotechnologie und den Möglichkeiten, diese mit der Anwendung von definierten Co-Kulturen zu lösen. Die potenziellen Anwendungen werden je nachdem, ob ihre Produkte den Energieträger ATP liefern, in zwei Kategorien unterteilt: dissimilatorische und assimilatorische Produkte. Zu den dissimilatorischen Produkten, deren Herstellung aus der Kohlenstoffquelle der Zelle ATP liefert, gehören Produkte wie Bioethanol. Aufgrund des großen Produktionsvolumens dieser Produkte können bereits geringfügige Ertrags- oder Produktivitätssteigerungen hohe wirtschaftliche Vorteile mit sich bringen. In dem Kapitel wird beschrieben, wie durch den Einsatz definierter Co-Kulturen in diesen Verfahren Nebenprodukte in Produkte umgewandelt und so die Produkterträge gesteigert werden können. Co-Kulturen von auf bestimmte Zucker spezialisierten Stämmen können außerdem eine allmähliche Abnahme der Fermentationsgeschwindigkeit vermeiden, der oftmals in Monokulturen von „Generalistenstämmen“ in Substratgemischen beobachtet wird. Bei der Herstellung assimilatorischer Produkte, zu denen komplexe Pflanzenmoleküle, Farbstoffe oder Duftstoffe zählen, müssen häufig komplexe Stoffwechselwege in die Zelle integriert werden, welche mit dem Wirtsorganismus inkompatibel sein können. Co-Kulturen verschiedener Produktionsorganismen sind hier ein Lösungsansatz und bieten außerdem eine potenzielle Ertragssteigerung durch das Beseitigen von Nebenprodukten. Co-Kulturen bieten vielversprechende Möglichkeiten, bergen aber auch neue Herausforderungen, wie zum Beispiel neue, unerwünschte Wechselwirkungen zwischen den Mitgliedern der Co-Kultur und die Notwendigkeit, die relative Menge der Produktionsstämmen während des Prozesses auf einem optimalen Niveau zu halten.

Eine andere Herausforderung tritt bei der Verwendung von Co-Kulturen von auf bestimmte Zucker spezialisierten *Saccharomyces cerevisiae*-Stämmen zur Verwertung von Substratgemischen auf und hängt mit Ungleichgewichten in der Wachstumskinetik der Stämme zusammen. Zwar bieten Co-Kulturen bei der Verstoffwechselung von Lignocellulose-Hydrolysaten Effizienzvorteile gegenüber Generalistenstämmen, die die verfügbaren Zucker nacheinander statt gleichzeitig verwerten, doch wird die Gesamtumwandlungszeit häufig vom langsamsten Fermenter bestimmt, bei dem es sich oft um einen Pentose-fermentierenden Stamm handelt. **Kapitel 2** beschreibt eine Strategie, ein solches Ungleichgewicht

bei sequenziellen Batch-Fermentationen zu überwinden. Bei sequenziellen Batch-Fermentationen wird das Wachstumsmedium nach Substratverbrauch regelmäßig durch neues Medium ersetzt, was die Möglichkeit bietet, vor dem Mediaustausch eine Biomasse-Sedimentierungsphase in den Prozess zu integrieren. Durch die Inaktivierung des Genes *ACE2* in einem langsam wachsenden, diploiden, Arabinose-fermentierenden *S. cerevisiae*-Spezialistenstamm wurde dieser so modifiziert, er schnell sedimentierende, multizelluläre Cluster bildet. Dies ermöglichte eine selektive Zunahme der Biomassekonzentration dieses Stammes während der Sedimentationsphasen und verbesserte die Kinetik in den nachfolgenden Batch-Zyklen. Die Strategie reduzierte die Gesamtumwandlungszeit in Monokulturen um 30 % und ermöglichte einen nahezu gleichzeitigen Verbrauch von Arabinose und Glukose in Co-Kulturen mit einem Glukose-Spezialistenstamm. Diese Studie zeigt, wie die Entwicklung eines multizellulären Phänotyps die Wachstumskinetik von Co-Kulturen in lang andauernden sequenziellen Batch-Fermentationen ausgleichen kann.

Um die Stabilität definierter Co-Kulturen zu steigern und das Aussterben einzelner Stämme zu verhindern, können kooperative Wechselwirkungen durch Nährstoffaustausch zwischen den Stämmen integriert werden. Solche Wechselwirkungen begünstigen jedoch häufig das Wachstum unproduktiver „Cheater“-Stämme, die ausgetauschte Nährstoffe abfangen und so die empfindliche Wechselwirkung stören. Unter räumlich definierten Bedingungen, z. B. auf Agarplatten, können solche Cheater-Populationen dadurch das Wachstum der Gesamtkultur beeinträchtigen. In **Kapitel 3** werden, durch eine Kombination aus theoretischen und experimentellen Ansätzen, die Faktoren untersucht, die dem Erfolg der kooperierenden Stämme in diesen Umgebungen zugrunde liegen. Es zeigte sich, dass ein einzelner Parameter – der Zeitpunkt des ersten Kontakts – das Wachstum der kooperierenden Stämme und damit der Gesamtkultur bestimmt. Dieses einfache Modell ist in der Lage, die zugrundeliegenden Dynamiken dieses hochkomplexen Systems zu erfassen und kann so wichtige Erkenntnisse liefern, die zum Verständnis der Dynamik zwischen Kooperatoren und Cheatern beitragen. Diese Erkenntnisse lassen sich auf biotechnologische Anwendungen übertragen, welche auf komplexen, ökologischen Wechselwirkungen beruhen, wie beispielsweise Joghurt- oder Kombucha-Konsortien.

Als Alternative zur rationalen Entwicklung von Co-Kulturen können Mikrodroplets als Screening-Werkzeug eingesetzt werden. Dies erlaubt es Forschern, mithilfe der Einkapselung einzelner Zellen oder Co-Kulturen in Pikoliter-große Mikrotröpfchen Bibliotheken von bis zu  $10^{10}$  Kandidaten pro Milliliter Volumen zu untersuchen. Durch diese Technik kann verhindert werden, dass die am schnellsten wachsenden Kandidaten langsamere, ertragreichere Stämme verdrängen. Da sich die Droplets jedoch in unmittelbarer Nähe zueinander befinden, hängt dieser Ansatz von

der vollständigen Kompartimentierung aller verfügbaren Metaboliten ab, um zu verhindern, dass diese in benachbarte Droplets übergehen und andere Zellen beeinträchtigen. In **Kapitel 4** wird beschrieben, dass eine solche Isolierung für einige biologisch relevante Moleküle, wie Ethanol oder schwache Säuren, nicht garantiert werden kann. Insbesondere Alkohole diffundieren aufgrund ihres amphiphilen Charakters zwischen den Tröpfchen, was sich auf die Ertragsberechnungen von Zellen auswirken kann, die diese Metaboliten verwerten können. Andere Metaboliten mit hoher Wasserlöslichkeit, wie zum Beispiel Zucker, verbleiben innerhalb der Tröpfchen. Auf Basis der unterschiedlichen Kompartimentierung der untersuchten Moleküle wird der Octanol-Wasser-Verteilungskoeffizient  $\log P$  als physikalischer Parameter zur Vorhersage des potenziellen Übertragungsrisikos identifiziert. In dieser Studie werden Richtlinien für die Auswahl von Substraten und Produkten festgelegt, um übertragungsfreie Screening-Setups zu gewährleisten.

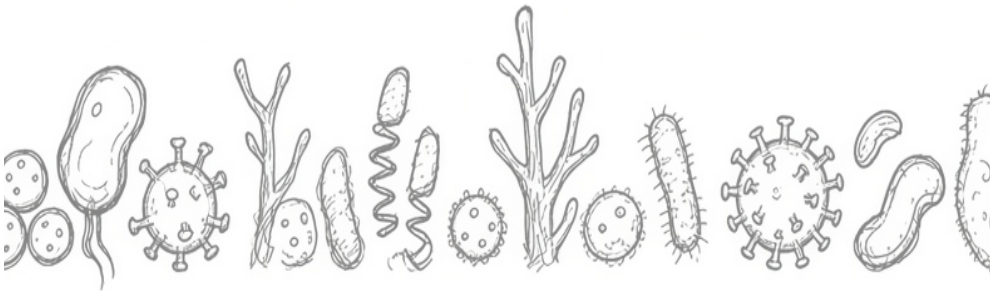
Diese Arbeit beleuchtet das Anwendungspotenzial mikrobieller Co-Kulturen in der mikrobiellen Biotechnologie. Durch eine Kombination von Gentechnik, ökologischer Modellierung und der Entwicklung von Screening-Tools werden praktische Strategien für die Entwicklung und die Verfahrensintegration dieser Kulturen vorgestellt. Diese Fortschritte können dabei helfen, den Weg für eine breitere Nutzung mikrobieller Co-Kulturen in biotechnologischen Anwendungen zu ebnen.

What are these pines & birds about?

What is this pond a-doing?

I must know a little more.

Henry David Thoreau, Journal entry April 19, 1852



# Chapter 1

## **Unlocking the potential of defined co-cultures for industrial biotechnology: opportunities and challenges**

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Essentially as published in New Biotechnology (2025) 91:89-99

<https://doi.org/10.1016/j.nbt.2025.11.009>

## **Abstract**

Large-scale microbial-biotechnology processes for production of chemicals almost exclusively rely on pure cultures of microbial strains. Especially for extensively engineered pure cultures, process performance can be negatively affected, which can be caused by issues such as pathway imbalance, deterioration of productivity caused by genetic instability and enzyme promiscuity. An increasing number of studies demonstrate that, under ‘academic’ laboratory conditions, the use of defined co-cultures (i.e. deliberate mixtures of known microbial strains) offers unique possibilities for mitigating such drawbacks. These advantages differ for dissimilatory products, whose synthesis from one or more carbon substrates provides cells with free energy, and assimilatory products, whose synthesis requires a net input of free energy. Based on advances in experimental and theoretical research, this paper highlights how defined co-cultures can address several limitations of mono-cultures for production of low-molecular-weight compounds. From this largely academic perspective, we outline the key challenges for scaling these systems to industry, which underscore the need for innovative solutions and continued research in this area.

## 1.1 Introduction

In industrial biotechnology, production processes traditionally use single microbial strains (monocultures). While in many cases effective, such monocultures can suffer from problems such as genetic instability resulting in loss of productivity, pathway imbalance and incompatibility of envisioned production strains with the functional expression of key enzymes. Defined co-cultures, i.e. mixtures of known microbial strains, have been proposed as a means to address these challenges.

A large body of knowledge generated in microbial ecology underscores the importance of microbial interactions for the functionality and stability of natural microbial communities [1–3]. Microbial co-cultures are widely applied in food fermentation processes, to enhance product quality parameters such as nutritional value and flavor [4–6]. Underlying metabolic interactions in these processes include commensalism (one species benefits and the other is neither harmed nor helped), mutualism (both species benefit) and parasitism (one species benefits and the other is not harmed) [7]. In contrast to food fermentation processes, industrial biotechnology, which uses microorganisms for production of chemicals, only rarely makes use of co-cultures of different strains or species [8,9]. Instead, it predominantly relies on monocultures of microbial strains, whose performance in industrial processes is often extended or improved by classical strain improvement and/or genetic engineering.

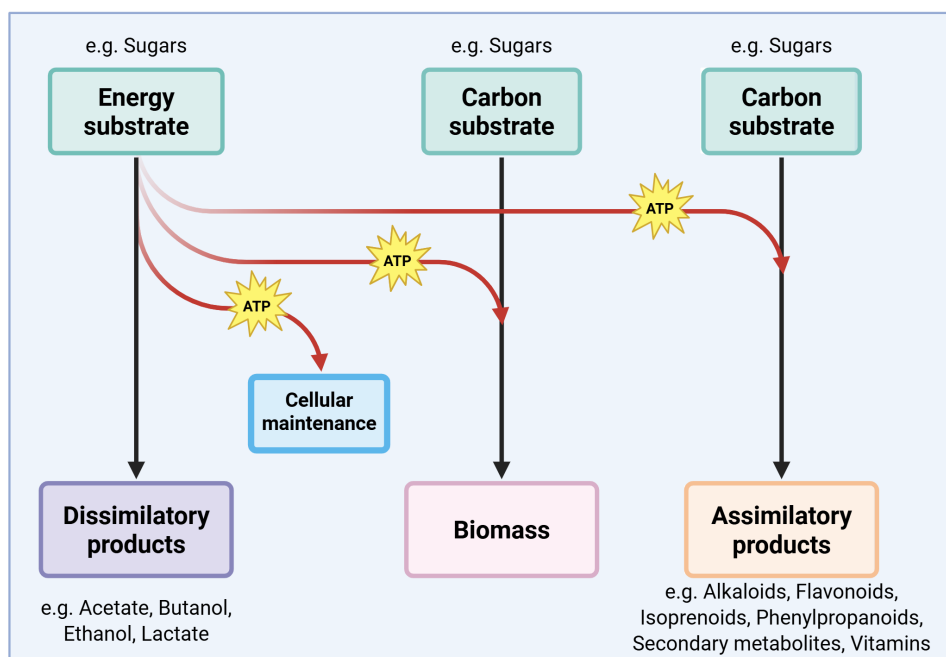
Products of industrial biotechnology range from fragrances and pharmaceuticals to transport fuels and chemical building blocks. Based on their connection to microbial energy metabolism, products of industrial biotechnology can be divided into two distinct categories: dissimilatory and assimilatory products. Figure 1.1 depicts both categories schematically. Since the advantages of co-cultures differ for these products, this review is split into two main parts: one around dissimilatory and one around assimilatory products. Dissimilatory products are compounds whose synthesis from a carbon substrate provides the cell with ATP. Examples include fermentation products such as ethanol and lactate, whose large-scale production typically occurs in anaerobic or oxygen-limited batch cultures. Assimilatory products are compounds whose synthesis from a carbon substrate requires a net investment of ATP and biosynthetic precursors. Therefore, in contrast to dissimilatory product formation, assimilatory product formation competes for ATP with cellular growth and maintenance. Assimilatory products produced by microorganisms include a wide and growing range of molecules, for which the biosynthetic pathways have often been introduced and/or improved by extensive genetic engineering. Examples of assimilatory products include structurally diverse molecules such as alkaloids, terpenoids, fatty acids, flavonoids, phenylpropanoids, and polyketides. To efficiently generate the ATP required for product formation, they are typically manufactured in aerobic fed-batch or continuous fermentation processes.

The TRY acronym (titer, rate, yield) captures three key performance indicators of industrial biotechnology processes [10]. Titer refers to the final concentration of the product ( $c_p$ , kg product per  $m^3$ ), which is highly relevant for efficient product purification by processes such as distillation or chromatography. Moreover, titer impacts the required bioreactor volume and, thereby, required capital investments. Rate can be expressed as productivity of the entire reactor ( $R_p$ , kg product per hour). Volumetric productivity ( $r_p$ , kg product per  $m^3$  per hour) links productivity to installed reactor volume and is the product of the biomass concentration ( $c_x$ ) and the biomass specific-production rate ( $q_p$ , kg product per kg biomass per hour). Processes with a higher volumetric productivity  $r_p$  require lower capital investments in installed bioreactor volume. The third key performance indicator is the product yield on substrate ( $Y_{p/s}$ , kg of product formed per kg of consumed substrate), which is equal to the ratio of the product-formation and substrate-consumption rates ( $Y_{p/s} = R_p/R_s = r_p/r_s = q_p/q_s$ ). The  $Y_{p/s}$  has a particularly strong impact on process economy for commodity products, for which feedstock costs can make up a large fraction of the overall production costs. For example, costs of the carbohydrate feedstock can account for up to 70% of the costs of yeast-based ethanol production [11].

Genetic stability of high-performance cell factories can be an additional key performance indicator in large-scale applications. Industrial fermentation processes for assimilatory processes are operated at reactor volumes of up to several hundred  $m^3$ , while bioreactor volumes for ethanol fermentation can even run into several thousand  $m^3$ . This implies that the ‘seed train’ needed to scale up from milliliter-scale frozen culture samples encompasses many generations during which a strain’s full production capacity needs to be maintained. Genetic stability is even more important when fermentation processes are operated continuously or as serial batch cultures with the aim to improve process economics and/or sustainability.

The aim of this paper is to review results from fundamental, application-inspired studies aimed at investigating if and how the use of defined microbial co-cultures can circumvent or mitigate limitations of monocultures as production systems. It primarily discusses results from studies that generated quantitative information on parameters such as titer, rate, yield and genetic stability. In addition, it focuses on production of low molecular weight compounds. Discussion of high molecular weight compounds (e.g. proteins and biopolymers), and their production processes (including consolidated bioprocessing, which combines enzyme production and fermentation in one step [12–16]), is therefore outside the scope of this review. The different relationships of assimilatory and dissimilatory product formation with cellular energy metabolism (Figure 1.1) generate different challenges in monocultures and, consequently, provide different opportunities for the use of defined co-cultures. We therefore separately discuss co-culture strategies for dissimilatory

and assimilatory product formation. These examples are summarized in Table 1.1. Based on this information, we subsequently outline key challenges that need to be addressed to enable broader use of defined co-cultures in industrial biotechnology. Together, these insights underline the potential of defined co-cultures to further develop and improve industrial biotechnology.



**Figure 1.1: Dissimilatory and assimilatory products.** Dissimilatory products are compounds whose synthesis from (a) substrate(s) provides the cell factory with ATP, which can be used for growth and cellular maintenance. Synthesis of assimilatory products from (a) substrate(s) requires a net ATP input and is therefore dependent on dissimilatory pathways to generate ATP. Created in BioRender. <https://BioRender.com/lf23uka>

Table 1.1: Selection of references on defined microbial co-cultures for industrial biotechnology, for which titer, rate, yield and/or stability of strain performance are quantitatively described.

| Product   | Substrate(s)       | Target                                                                            | Strategy                                                                                                                                                                                                    | Titer/Rate/Yield of co-culture (monoculture between brackets)               | Ref. |
|-----------|--------------------|-----------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------|------|
| Ethanol   | Glucose            | Increase product yield on substrate through conversion of by-products to product. | Co-culture of PRK-RuBisCo based and A-ALD-based <i>S. cerevisiae</i> strains, where the A-ALD strain converts by-products from the PRK-RuBisCo strain (acetate and acetaldehyde).                           | 1.67 (1.64, PRK-RuBisCo based strain) g ethanol · (g glucose) <sup>-1</sup> | (17) |
|           | Glycerol           | Increase product yield on substrate through conversion of by-products to product. | Co-culture of <i>Klebsiella spp</i> and <i>Shewanella oneidensis</i> , where <i>S. oneidensis</i> oxidizes the by-product lactate from <i>Klebsiella spp</i> supplying electrons for its product formation. | 0.44 (0.41) g 1,3-PD · (g glycerol) <sup>-1</sup>                           | (18) |
| Lactate   | Glucose and xylose | Increase volumetric productivity through improved sugar utilization kinetics.     | Co-culture of two <i>E. coli</i> strains, each specialized in glucose and xylose consumption.                                                                                                               | 5.8 (2.6) g lactate · L <sup>-1</sup> · h <sup>-1</sup>                     | (19) |
| n-Butanol | Glucose and xylose | Increase volumetric productivity through improved sugar utilization.              | Co-culture of two <i>E. coli</i> strains, each specialized in glucose and xylose consumption.                                                                                                               | 0.12* (0.072*) g n-butanol · L <sup>-1</sup> · h <sup>-1</sup>              | (20) |

|                              |                               |                                                                                                                   |                                                                                                                                                       |                                                                                 |      |
|------------------------------|-------------------------------|-------------------------------------------------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------|------|
| Ethanol                      | Arabinose, glucose and xylose | Improved genetic stability during long term cultivation on sugar mixture.                                         | Co-culture of three <i>S. cerevisiae</i> strains, each specialized in arabinose, glucose and xylose consumption.                                      | Cycle length: stable at 35 h for 1000 h cultivation (increase from 25 h to 51h) | (21) |
| Rosmarinic acid              | Glucose                       | Increase product titer by pathway modularization.                                                                 | Employ engineered three-strain <i>E. coli</i> co-culture to balance complex diverging-converging biosynthetic pathway by improved metabolic flux.     | 74 (4.5) mg·L <sup>-1</sup>                                                     | (22) |
| Caffeyl alcohol              | Glucose                       | Increase product titer by spatial separation to avoid negative impact of enzyme promiscuity.                      | Co-culture of p-CA-producing and HpaBC-expressing <i>E. coli</i> strains to prevent L-dopa by-product formation due to HpaBC promiscuity.             | 0.4 (0.03) g·L <sup>-1</sup>                                                    | (23) |
| Eugenol                      | Glycerol and glucose          | Increase product titer by preventing intermediate toxicity and enzyme promiscuity through pathway modularization. | Tripartite <i>E. coli</i> co-culture separating coumarate, ferulic acid, and eugenol modules to avoid substrate competition and toxic build-up.       | 0.07 (0) g·L <sup>-1</sup>                                                      | (24) |
| Anthocyanidin-3-O-glucosides | Glucose                       | Increase product titer of anthocyanin through pathway modularization.                                             | Four-strain polyculture distributing 15 pathway enzymes across modules to reduce metabolic burden and enable <i>de novo</i> anthocyanin biosynthesis. | ~ 9.5* (0*) mg pelargonidin-3-O-glucoside ·L <sup>-1</sup>                      | (25) |

|                        |                    |                                                                                                                    |                                                                                                                                                                                                                |                                 |      |
|------------------------|--------------------|--------------------------------------------------------------------------------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------|------|
| Oxygenated taxanes     | Xylose and acetate | Extend product range and improve product titer by addressing host-specific enzyme functional expression challenge. | Modular co-culture of <i>E. coli</i> producing taxadiene and <i>S. cerevisiae</i> expressing plant P450 fusion protein to support oxygenation steps in taxane biosynthesis.                                    | 0.03 (0) g·L <sup>-1</sup>      | (26) |
| Various strigolactones | Glucose            | Extend product range and improve product titer by addressing host-specific enzyme functional expression challenge. | Bipartite <i>E. coli</i> and <i>S. cerevisiae</i> co-culture: <i>E. coli</i> produces carlactone (3 enzyme-types), while yeast expresses downstream P450 enzymes for conversion to specific strigolactones.    | Up to 47 (0) µg·L <sup>-1</sup> | (27) |
| (S)-Norcoclaurine      | Glucose and xylose | Improve product titer by binary modularization of two-species co-culture and module optimization.                  | <i>Schefferomyces stipitis</i> produces shikimate from mixed sugars; <i>S. cerevisiae</i> , engineered with <i>Aspergillus</i> shikimate transporters, imports shikimate and converts it to (S)-norcoclaurine. | 11.5 (0.1) mg·L <sup>-1</sup>   | (28) |

\*Values calculated based on information in figures or in text.

## 1.2 Dissimilatory products

The large product volumes and narrow profit margins of dissimilatory products [10] imply that small improvements of titer, rate and/or yield can already confer significant economic benefits. Improving product yield on substrate ( $Y_{p/s}$ ), for example by reducing by-product formation, is especially important because substrate costs strongly influence process economics [10,11]. Recycling of biomass from one fermentation process to the next can help to improve volumetric productivity ( $r_p$ ) [29]. When contamination by undesirable competing microorganisms is kept at bay, such biomass recycling strategies can work very well because dissimilatory product formation from a single substrate is linked to growth and survival and therefore confers a selective advantage to fast-producing mutants [30,31]. To improve process sustainability and reduce land use for production of fermentation feedstocks, conversion of sugar mixtures generated by hydrolysis of lignocellulosic agricultural residues is intensively explored [32,33]. Below, we will discuss examples of how the use of defined co-cultures has been explored as a means to (i) improve  $Y_{p/s}$  by minimizing by-product formation (section 1.2.1), (ii) improve volumetric productivity ( $r_p$ ) during conversion of sugar mixtures (section 1.2.2) and (iii) improve genetic stability in repeated batch cultures grown on sugar mixtures (section 1.2.3). These scenarios are illustrated in Figure 1.2 a-c.

### 1.2.1 Minimizing by-product formation to improve yield

Ethanol production with the yeast *Saccharomyces cerevisiae* is the largest-volume process (ca. 100 Mton per year [31]) in industrial biotechnology. In anaerobic cultures of this yeast, including the large-scale batch cultures used for industrial ethanol production, glycerol formation serves as essential ‘redox sink’ for re-oxidation of a surplus of NADH generated in biosynthetic reactions. Without mitigating measures, glycerol formation would account for a loss of 4% of the carbohydrate feedstock used for yeast-based ethanol production [34].

A metabolic engineering strategy to eliminate glycerol formation was based on functional expression of heterologous genes encoding the two signature enzymes of the Calvin cycle for CO<sub>2</sub> fixation, phosphoribulokinase (PRK) and ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco). Introduction of functional PRK and Rubisco in *S. cerevisiae* enabled a redox-cofactor-neutral conversion of glucose to 3-phosphoglycerate, whose subsequent NADH-dependent conversion to ethanol and CO<sub>2</sub> could replace the role of glycerol as redox sink. In fast-growing, glucose-grown anaerobic batch cultures, this strategy enabled an over 10% higher  $Y_{p/s}$  [35]. However, at suboptimal growth rates, which occur in the later stages of industrial processes due to ethanol accumulation and depletion of non-sugar nutrients, an overcapacity of the engineered PRK-Rubisco bypass led to formation of the

by-products acetaldehyde and acetate [36]. An alternative metabolic engineering strategy for coupling reoxidation of 'excess' NADH to ethanol formation was based on introduction of a heterologous acetylating acetaldehyde dehydrogenase (A-ALD) [37]. Together with native yeast enzymes, A-ALD enables the NADH-dependent reduction of acetate to ethanol. However, the concentration of acetate in 'first-generation' feedstocks for ethanol production such as corn starch hydrolysates is too low to completely replace glycerol production in acetate-reducing yeast strains. Co-cultivation of an engineered PRK/Rubisco-based and an A-ALD-based strain was shown to combine the advantages of the two strains: low concentrations of acetate present in the medium, as well as acetaldehyde and acetate generated by the PRK/Rubisco strain, were efficiently converted to ethanol by the A-ALD strain, while the PRK/Rubisco strain supported acetate- and acetaldehyde-independent, high-yield ethanol production. Optimization of the inoculation ratio of this commensalistic co-culture enabled overall fermentation times equal to those of monocultures of the PRK/Rubisco strain [17].

Wang *et al.* [18] explored a strategy for channelling by-products into product formation that involves two bacterial species. Anaerobic cultures of the *Klebsiella* strain used in this study produced 1,3-propanediol (1,3-PD) from glycerol, a conversion that requires a net input of electrons in the form of NADH. In the *Klebsiella* strain, this NADH is made available by converting part of the glycerol to more oxidized by-products such as acetate and lactate [38], which limits the maximum yield of 1,3-PD on glycerol. To mitigate by-product formation, the *Klebsiella* strain was co-cultivated with an exo-electrogenic *Shewanella oneidensis* strain. The rationale of this co-cultivation strategy was that *S. oneidensis* can oxidize lactate, generated by the *Klebsiella* strain, to acetate and CO<sub>2</sub> and transfer the electrons that are released during this oxidation to the *Klebsiella* strain. This interspecies electron transfer may occur either via exported FMNH<sub>2</sub> and reduced riboflavin or via direct interspecies electron transfer through pili [18,39]. The *Klebsiella* strain then uses the electrons for reducing glycerol to 1,3-PD. Consistent with this mutualistic mechanism, co-cultivation of the two bacteria led to a 7.3% higher yield of 1,3-PD on glycerol than observed in monocultures of the *Klebsiella* strain.

Overall, these examples show that co-cultures can be used to convert by-product into product, thereby improving product yield on substrate.

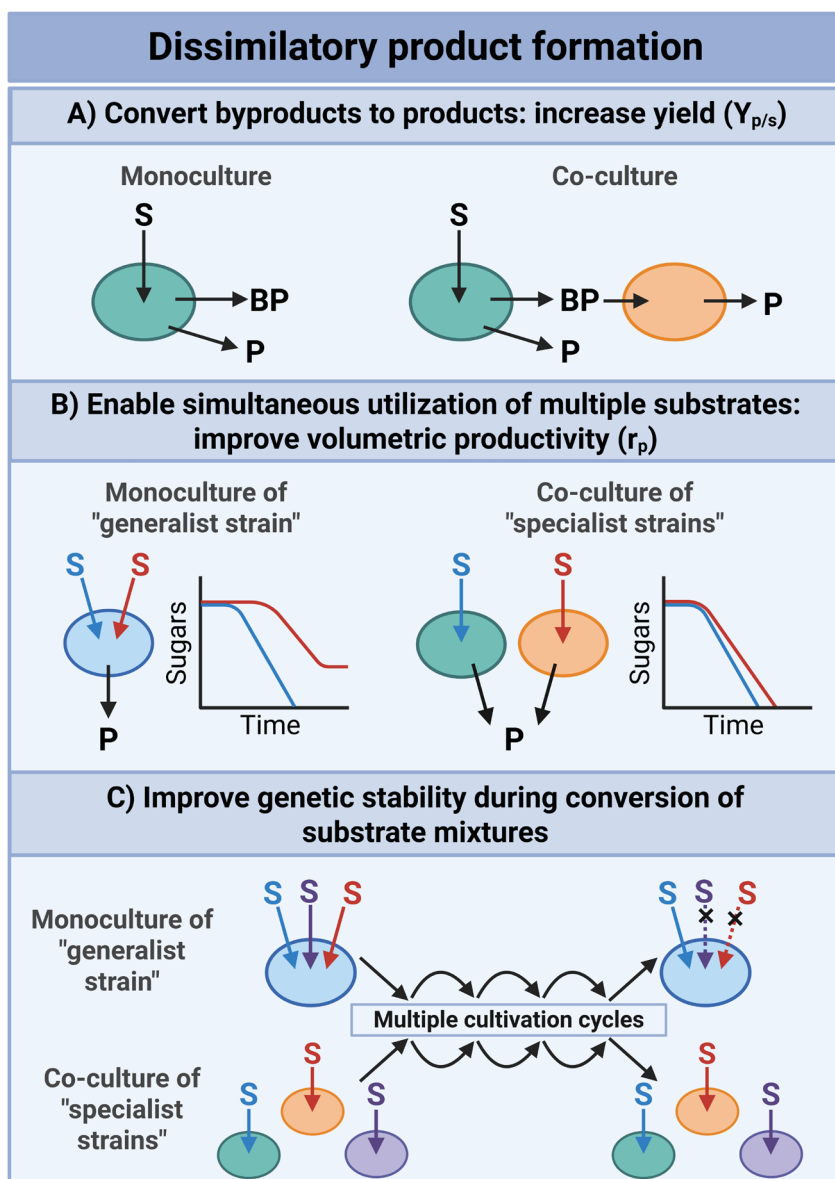


Figure 1.2: Potential benefits of using defined co-cultures to improve titer, rate, yield and stability during production of low molecular weight dissimilatory products. a) In defined co-cultures, microorganisms can utilize or sequester by-products formed by other microorganisms to increase overall product yield from substrate. b) Defined co-cultures of specialist strains (utilizing a single substrate) could allow for a higher consumption rate of each substrate due to preferential substrate utilization by the generalist strain. c) Generalist strains are more likely to become genetically unstable after multiple cultivation rounds on substrate mixtures than specialist strains, resulting in diminished substrate utilization capabilities. S = substrate; P = product; BP = By-product. Created in BioRender. <https://BioRender.com/zsu15iq>

## 1.2.2 Improving mixed-substrate utilization

Combined chemical and enzymatic hydrolysis can release fermentable sugars from agricultural residues such as corn stover and sugar cane bagasse, as well as from 'energy crops' such as switchgrass [32,33]. Use of such 'second-generation' feedstocks offers a large potential for improving the carbon footprint of industrial biotechnology. However, in contrast to feedstocks such as cane sugar or hydrolysed corn starch, these feedstocks contain mixtures of multiple sugars, with glucose and two pentose sugars, xylose and arabinose as main contributors. Industrial microorganisms such as *S. cerevisiae* and *Escherichia coli* have been extensively engineered to construct 'generalist' strains that convert mixtures of these three sugars. While these strains achieve high product yields on the sugar mixture under anaerobic conditions, their fermentation kinetics are often suboptimal. Engineered strains typically first consume the glucose at a high rate, while pentoses are consumed in a second, much slower sugar fermentation phase, thereby reducing the volumetric productivity  $r_p$  [21,40].

Generalist strains need to simultaneously synthesize enzymes for multiple substrate-conversion pathways. Monod pioneered research on diauxic growth, which is the sequential use of preferred and less-preferred substrates when these are provided together in batch cultures [41]. The resulting bi-phasic growth illustrates how natural evolution yielded regulation mechanisms that, in the presence of non-limiting concentrations of multiple substrates, prevent simultaneous allocation of cellular resources to multiple substrate-utilization pathways. Instead, microorganisms have evolved to maximize growth rate by allocating cellular resources to fast conversion of a single substrate at a time [42]. This principle led to multiple application-inspired studies, in which fermentation kinetics of defined co-cultures of 'substrate specialist strains' in cultures grown on sugar mixtures were compared with those of monocultures of a generalist strain.

Flores *et al.* [19] compared fermentation kinetics in aerobic cultures on a glucose-xylose mixture of an *E. coli* generalist strain capable of converting glucose and xylose, with those of a bipartite co-culture of glucose- and xylose-specialist *E. coli* strains grown on the same medium. An over 2-fold higher volumetric productivity of lactate was observed in the co-cultures than in cultures of the generalist strain (5.8 g lactate L<sup>-1</sup> h<sup>-1</sup> versus 2.6 g L<sup>-1</sup> h<sup>-1</sup>). In a conceptually similar study, Saini *et al.* [20] compared product formation in anaerobic cultures, grown on a glucose-xylose mixture, of a generalist *E. coli* strain and a co-culture of two sugar-specialist strains, all engineered for n-butanol production. In a 36-h growth experiment, the generalist strain produced 2.6 g L<sup>-1</sup> n-butanol and converted only about 60% of the available sugars. Over the same period, the co-culture produced 4.4 g L<sup>-1</sup> n-butanol and achieved near-complete sugar conversion [20]. The improved productivities

observed in these studies are consistent with an improved protein allocation in the co-culture and/or elimination of a pathway interference in generalist strains.

The above examples, as well as additional studies [43,44], show that the use of co-cultures of specialist strains can improve production rates during conversion of mixed substrates.

### 1.2.3 Enhancing genetic stability during sequential batch cultivation on mixed substrates

Dissimilatory product formation can, via generation of ATP (Figure 1.1), be directly coupled to growth rate. This principle has been extensively used to increase product formation rates by adaptive laboratory evolution in serial-batch monocultures [30]. Selection for improved fermentation kinetics has also been documented for the Brazilian bioethanol industry, in which ethanol production from cane sugar involves recycling of yeast biomass from one culture to the next over multi-month campaigns [31]. In contrast to these observations on single-substrate cultures, studies in which serial-batch monocultures of 'generalist' strains were grown on mixtures of carbon substrates, reported a deterioration of overall fermentation kinetics (Figure 1.2b and 1.2c, [21,45,46]). An *S. cerevisiae* strain engineered for efficient conversion of glucose, xylose and arabinose showed a progressive increase of overall fermentation times when subjected to sequential batch cultivation cycles on mixtures of xylose and arabinose [45] or glucose, xylose and arabinose [21,46].

In a batch culture grown on equivalent concentrations of multiple substrates, the largest number of generations occurs during growth on the most preferred substrate. In serial batch cultures, selective pressure for faster utilization of a substrate is proportional to the number of generations of growth on that substrate. In cultures grown on substrate mixtures, competition for cellular resources or other interference of pathways will therefore preferentially select for faster utilization of the already favored substrate, at the expense of growth rates on the less preferred substrates. The resulting evolution towards substrate specialization rather than towards co-utilization presents a challenge in developing biomass-recycling strategies for the industrial fermentation of sugar mixtures with monocultures of generalist strains.

A co-culture of specialist strains that can each ferment only a single substrate in a mixture is not expected to show deterioration of fermentation kinetics during serial batch cultivation on substrate mixtures. Instead, in such cultures, each strain is anticipated to experience selective pressure to improve growth rate on its 'assigned' sugar. This concept was experimentally tested by Verhoeven *et al.* [21], who used metabolic engineering and adaptive laboratory evolution to obtain specialist *S. cerevisiae* strains that were able to anaerobically ferment xylose or arabinose when grown on a mixture of glucose, xylose, and arabinose. A non-engineered strain of

1 *S. cerevisiae* that cannot ferment pentose sugars was used as glucose specialist. A consortium of the three specialist strains was grown in anaerobic sequential batch cultures on glucose, xylose and arabinose, whose relative concentrations were chosen to mimic those in lignocellulosic hydrolysates. In a control experiment with a generalist strain, overall fermentation time increased from approximately 25 h in the first cycle of sequential batch fermentation to over 50 h in the 24<sup>th</sup> cycle (Figure 1.2b). In contrast, over a similar number of cycles, fermentation kinetics of co-cultures of the three specialist strains improved rather than deteriorated [21].

While confirming that the use of specialist strains circumvents the degeneration of fermentation kinetics that was observed in monocultures of a generalist strain, the study of Verhoeven *et al.* [21] also identified a potential trade-off. The strong preference of the specialist strain for glucose led to a rapid build-up of biomass. Despite the lower biomass-specific conversion rates of xylose and arabinose, this biomass build-up still enabled a high volumetric conversion rate of these pentoses after glucose had been consumed. Instead, in the consortium of specialist strains, the lower conversion rate of the pentoses led to a longer overall conversion time than observed for the generalist strain. While fermentation kinetics of the consortium improved during serial batch cultivation, the overall fermentation time after the 24<sup>th</sup> cycle was still ca. 20% slower than observed in the first cycles of serial batch cultivation of the generalist strain. This trade-off is influenced by the relative concentrations of the individual substrates, which determine the initial biomass concentration of each specialist strain upon initiation of the next growth cycle.

The above example shows that, in co-cultures, strains can be engineered to convert only one of the substrates in a substrate mixture. When producing a dissimilatory and therefore growth-coupled product, each specialist strains is under selective pressure to optimize its biomass specific productivity ( $q_p$ ). This selective pressure prevents the deterioration of fermentation kinetics that is seen during prolonged batch cultivation of generalist strains on sugar mixtures.

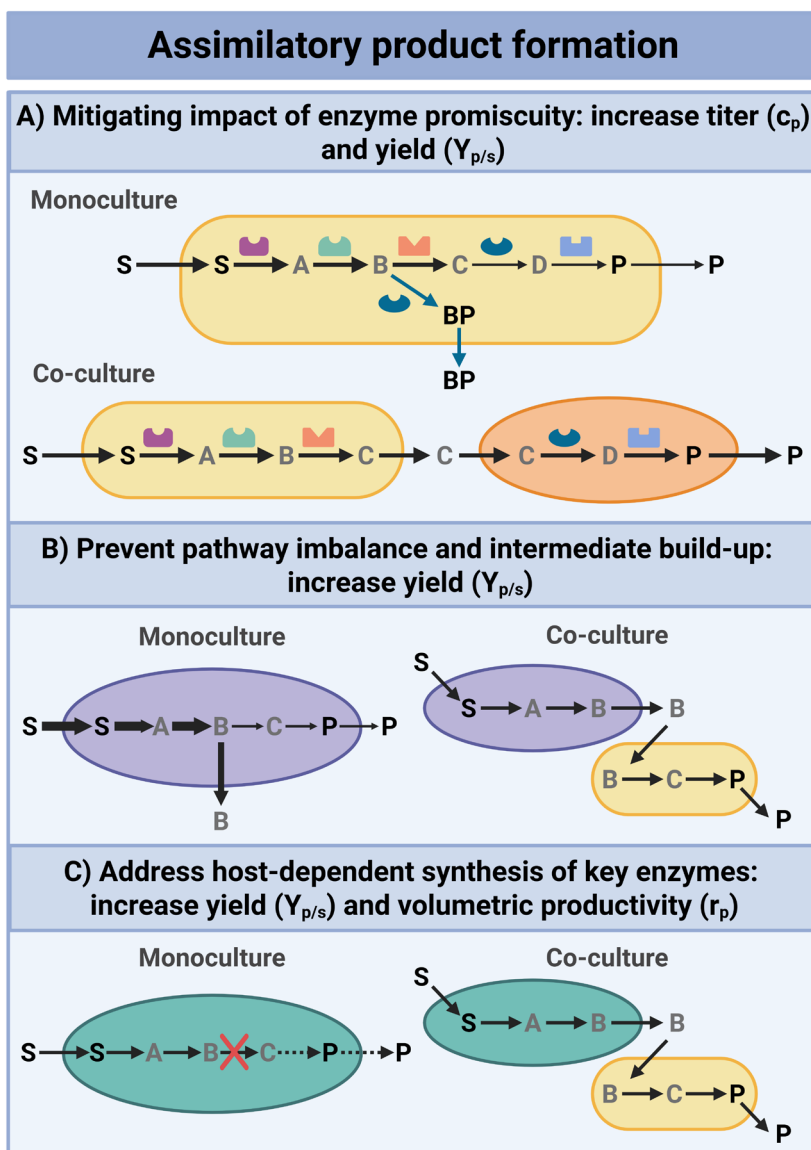
In summary, for dissimilatory processes, defined co-cultures can improve yield, substrate range, and long-term genetic stability by distributing metabolic tasks over specialized strains.

### 1.3 Assimilatory products

In comparison with dissimilatory products, assimilatory processes (whose synthesis requires a net energy input) face different challenges. These include competition of growth and product formation for cellular resources, long product pathways, and a need to synthesize complex, non-native enzymes. In these cases, co-cultures offer alternative options to improve process performance.

Introduction of metabolic pathways for synthesis of complex, non-native assimilatory products by microbial cell factories can require expression of dozens of genes, sourced from multiple donor organisms [47,48]. Producing all the encoded enzymes in a single engineered microorganism can be challenging. The umbrella term ‘metabolic burden’ is often used to capture negative effects of such intensive metabolic engineering on performance and genetic stability of the resulting strains [49,50]. Most studies on the use of co-cultures for assimilatory product formation aim to mitigate metabolic burden by spatial segregation of product-pathway modules in different microbial strains [51].

Native protein synthesis is already the most ATP-intensive process in growing, wild-type microbial cells [52]. Since, moreover, cell-volume and membrane-surface limitations impose constraints on cellular protein content [53,54], expression of heterologous pathways will compete with native cell functions for amino acids, ATP and proteome space. The impact of this competition is especially when product pathways involve enzymes that have a low catalytic turnover rate ( $k_{cat}$ ) and therefore need to be expressed at high levels to sustain relevant *in vivo* fluxes. The resulting negative impact on growth rate can confer a substantial selective advantage to less- or non-producing mutants [55]. It is often assumed that even distribution of pathway enzymes over different strains can alleviate this protein burden and, thereby, enable improved productivity of co-cultures [51,56]. However, based on theoretical considerations [57], equal distribution of pathway-related protein mass over multiple strains does not in itself enable a higher biomass-specific conversion rate than is obtained in a monoculture. Instead, unless equal distribution affects intracellular concentrations of pathway intermediates and/or effectors and thus enables a higher *in vivo* substrate saturation ( $V/V_{max}$ ) of rate-controlling enzymes, it will not influence the overall protein requirement. Achieving a given rate of product formation will then, per g of biomass, require the same amount of protein in mono- and co-cultures [58]. Transport of pathway intermediates between co-culture partners can decrease efficiency due to dilution of intermediates in the extracellular space and/or costs associated with the expression of transporters [51,57]. To have a net positive impact, co-cultivation strategies should therefore address metabolic burden issues that substantially constrain monoculture performance and cannot be addressed by straightforward metabolic engineering. Below, we will discuss examples of how the use of defined co-cultures can address challenges associated with catalytic promiscuity of pathway enzymes, by-product formation caused by pathway imbalance, and sub-optimal expression of key enzymes. Figure 1.3a, b and c schematically show the advantage of co-cultures for each of these challenges. In addition, we will discuss engineering of transport reactions, definition of metabolic modules and the relevance and control of the relative abundance of co-culture partners.



**Figure 1.3: Potential benefits of using co-cultures to improve titer, rate, yield and stability during production of low molecular weight assimilatory products.** a) Enzyme promiscuity can cause by-product formation in long heterologously expressed product pathways. Co-cultures could mitigate this reduction in product yield by splitting pathways over separate modules, thereby spatially segregating the promiscuous enzyme from its substrate. b) Co-cultures can be used to reduce the accumulation of (toxic) intermediates that would otherwise inhibit growth and lower product yield. c) Co-cultures of different species can be used to overcome host-specific limitations of enzyme expression. S = substrate; P = product; BP = By-product. Created in BioRender. <https://BioRender.com/nw7b4y7>

### 1.3.1 Addressing enzyme-related constraints in complex pathways

Enzyme promiscuity, i.e., the ability of an enzyme to perform different reactions [59], is a common challenge in the implementation of complex product pathways in industrial microorganisms [60,61]. The same holds for by-product formation caused by imbalances in engineered product pathways. Research on engineering of microbial cell factories for production of phenylpropanoids stimulated multiple studies on how these challenges can be mitigated by co-cultivation strategies.

Enzyme promiscuity can lead to by-product formation, which lowers the product yield on the substrate. This is particularly problematic for long heterologous pathways, as it creates side reactions that compete with the main product path. Co-cultures can offer a solution by separating the enzyme and the metabolite for which it has promiscuous activity (Figure 1.3a).

This enzyme promiscuity, for example, played a key role in a study on metabolic engineering of *E. coli* to produce monolignols [23]. Engineering of the native shikimate pathway for aromatic amino-acid synthesis, combined with introduction of a 5-step pathway for conversion of tyrosine to p-coumaryl alcohol (p-CA), enabled p-CA titers in shake-flask cultures of up to 0.5 g·L<sup>-1</sup>. However, extension of the p-CA pathway by overexpression of a 4-hydroxyphenylacetate-3-hydroxylase (HpaBC) that converts p-CA to caffeyl alcohol (CfA), yielded a CfA titer of only 0.03 g·L<sup>-1</sup>. This low titer was attributed to promiscuity of HpaBC which, in addition to the desired reaction, also converted tyrosine into the undesirable by-product L-dopa. Based on the observation that p-CA, but not tyrosine, easily crosses the *E. coli* cell membrane, the p-CA-producing strain was co-cultivated with a strain that overexpressed HpaBC in an otherwise wild-type background. After optimization of the inoculum ratio of the two strains, a CfA titer of 0.4 g·L<sup>-1</sup> was achieved in shake-flask-grown commensalistic co-cultures, indicating that expression of the promiscuous HpaBC enzyme in a separate strain successfully prevented by-product formation [23].

Brooks *et al.* [24] implemented a pathway in *E. coli* for production of the plant metabolite eugenol, in which tyrosine is first converted to coumarate, whose hydroxylation then yields caffeate. In the following two reactions, caffeate is first methylated to yield ferulic acid, which is then converted into feruloyl-CoA by the enzyme 4-hydroxycinnamoyl-CoA ligase (4-CL). However, in addition to ferulic acid, 4-CL can also use coumarate as a substrate [24,62], thereby diverting this key precursor from product formation. To address promiscuity of 4-CL and other enzymes in the eugenol pathway and to prevent build-up of the toxic intermediate coumarate, a tripartite co-culture was designed and constructed (Figure 1.3b). The first *E. coli* strain was engineered for efficient de novo production of coumarate (Module 1), a second strain for the two-step conversion of coumarate to ferulic acid (Module 2), and a third strain for the 5-step conversion of ferulic acid to eugenol

1 (Module 3). This modular design effectively separated 4-CL, which was highly expressed in Module 3, from upstream metabolites. In addition, increasing the inoculum size of the strain carrying Module 2 relative to that of the other two strains prevented accumulation of toxic levels of coumarate. A eugenol titer of  $0.07 \text{ g} \cdot \text{L}^{-1}$  was achieved in shake-flask cultures of the tripartite commensalistic co-culture, while no eugenol production was detected in a monoculture of an *E. coli* strain carrying all three modules. An additional advantage of the modular co-culture approach was demonstrated by further experiments, in which genetic modification or omission of Module 2 enabled production of chavicol and hydroxychavicol, respectively [24].

The above examples illustrate that by dividing the pathway between multiple strains, the promiscuous enzyme's side reaction was effectively bypassed, improving the yield of the desired product. Moreover, as also demonstrated by a study on the production of anthocyanins by tetrapartite *E. coli* co-cultures [25], the combination of strains optimized for precursor generation with 'terminal product formation' strains enables flexible 'mix and match' strategies for extending product range.

Pathway imbalances in a single strain can reduce product yields on substrate, for example by accumulation of intermediates. Balancing the activities of individual pathway enzymes is particularly challenging in the case of non-linear pathways, where optimal distribution of intermediates requires careful tuning of the *in vivo* activities of enzymes active at metabolic branchpoints. By separating pathways in different strains in a co-culture, these pathway imbalances can be relieved (Figure 1.3b). Li *et al.* [22] investigated this strategy using rosmarinic acid (RA) production by engineered *E. coli* strains as the experimental model. RA is formed by condensation of two molecules derived from the phenylpropanoid pathway: caffeoyl-CoA (Caf-CoA) and salvianic acid A (SalvA). Pathways towards Caf-CoA and SalvA diverge at 4-hydroxyphenylpyruvate (4-HPP). Conversion of 4-HPP to Caf-CoA starts with a two-step conversion of HPP into coumarate, after which two enzymes, including the abovementioned HpaBC hydroxylase, convert coumarate to Caf-CoA. Conversion of 4-HPP to SalvA is catalyzed by HpaBC and a D-lactate dehydrogenase. In this two-enzyme conversion, the order of the hydroxylase and reduction reactions is interchangeable. The ability of HpaBC to catalyze three reactions involved in the synthesis of two pathway intermediates presents a major challenge for pathway balancing in monocultures. This challenge was addressed by splitting RA biosynthesis into three pathway modules allocated to three different *E. coli* strains. Module 1 was engineered for *de novo* production of coumarate, Module 2 for *de novo* SalvA production and Module 3 for conversion of coumarate (generated by Module 1) to Caf-CoA and its condensation with SalvA (generated by Module 2) to yield RA. This tripartite co-culture strategy spatially segregated the roles of HpaBC in SalvA and Caf-CoA production and, by tuning the relative inoculum ratios of the three strains, presented a means to prevent build-up of coumarate to

toxic levels. After empirically optimizing the inoculum ratios, a tripartite co-culture yielded an RA titer of  $0.10 \text{ g}\cdot\text{L}^{-1}$  in glucose-grown shake-flask cultures, which was approximately 20-fold higher than the titer obtained with a monoculture carrying the entire RA pathway [22].

Overall, this example shows that using a commensalistic co-culture to separate different enzymes can prevent the accumulation of (toxic) intermediates, thereby enabling increased product yields on substrate.

Another challenge in mono-culture designs relates to host-dependent (dis)abilities to functionally express specific enzymes or enzyme classes. This problem can even rule out popular industrial microorganisms as production platforms. In such cases, using co-cultures with specialist strains of species that express the missing enzyme(s) can result in improved product yields and rates of product formation (Figure 1.3c). The potential of defined co-cultures for circumventing challenges related to expression of specific types of enzymes is illustrated by research on microbial production of oxyfunctionalized taxanes. Already in 2010 [26], *E. coli* was successfully engineered to produce taxadiene, a key precursor for biosynthesis of oxygenated taxanes, including the anticancer drug paclitaxel. However, synthesis of oxygenated taxanes from taxadiene depends on hydroxylases whose *in vivo* activity requires a cytochrome P450 reductase. *Escherichia coli* does not naturally contain P450 enzymes [63], and heterologous expression of these proteins can be challenging. Mostly due to its ability to anchor P450 proteins to intracellular membranes, *S. cerevisiae* is a preferred host for expressing this class of enzymes [64]. This inspired Zhou *et al.* [65] to study the co-cultivation of a taxadiene-producing *E. coli* strain with an *S. cerevisiae* strain that highly expressed a fusion protein of the plant taxadiene  $5\alpha$ -hydroxylase and P450 reductase that together catalyse the first oxygenation step in paclitaxel biosynthesis. Co-cultures of the two species were grown on xylose. Since xylose cannot be used by wild-type *S. cerevisiae*, growth of the yeast depended on consumption of acetate generated by the *E. coli* strain. After optimization of the expression of the hydroxylase-P450 reductase fusion protein in *S. cerevisiae* and of acetate production by *E. coli*, the titer of the targeted oxygenated taxane in fed-batch co-cultures reached  $0.03 \text{ g}\cdot\text{L}^{-1}$ . Introduction of expression cassettes encoding a second hydroxylase-P450 reductase protein and an acetylase into the *S. cerevisiae* strain enabled production of a next intermediate in the paclitaxel synthesis pathway. The versatility of this modular approach was further demonstrated by its application to the production of other compounds whose synthesis involves P450-dependent oxyfunctionalization reactions [65].

Wu *et al.* [27] demonstrated how, in bipartite co-cultures, engineered *E. coli* and *S. cerevisiae* strains could mutually complement species-specific limitations related to functional expression of key product-pathway enzymes. Their study focused

on microbial production of strigolactones, an important class of plant signalling molecules. Strigolactone synthesis starts from  $\beta$ -carotene, whose production has been demonstrated in engineered strains of *E. coli* and *S. cerevisiae* [66]. Three plant enzymes are required for conversion of  $\beta$ -carotene to carlactone, the central precursor for all strigolactones: a [2Fe-2S]-containing isomerase and two enzymes that catalyse oxidative cleavage reactions, one of which is a non-heme-iron-dependent enzyme. These three enzymes could be functionally expressed in *E. coli*, which enabled construction of a carlactone-producing strain. However, in line with previously reported challenges in cytosolic expression of heterologous iron-sulfur proteins in the yeast cytosol [67], attempts to produce carlactone in *S. cerevisiae* were unsuccessful. Conversely, the subsequent conversion of carlactone to specific strigolactones, which involves P450-dependent oxidation reactions, could be established in *S. cerevisiae* but not in *E. coli*. This inspired the authors to develop a flexible, bipartite co-cultivation strategy. Co-cultivating the carlactone-producing *E. coli* strain with a range of *S. cerevisiae* strains engineered for conversion of carlactone to specific strigolactones enabled production of the targeted compounds, thereby opening the way for studies on their biological functions [27].

The above examples illustrate that, when a specific pathway enzyme cannot be functionally expressed in a preferred industrial microorganism, co-cultivation with another microorganism that does functionally express the enzyme can expand the product range.

### 1.3.2 Definition of pathway modules and transport reactions

In implementing division-of-labour strategies for assimilatory product formation, as discussed in the previous paragraph, optimal design of metabolic modules is essential. Here, 'metabolic modules' refer to segments of a production pathway that are assigned to a single co-cultivation partner. By assigning different modules to different strains, each strain handles a part of the pathway. Design of functional modules should address primary objectives, such as mitigating effects of enzyme promiscuity, circumventing host-specific protein expression challenges and preventing pathway imbalance. A second criterium, is the availability of mechanisms for transport of relevant intermediates between co-cultivation partners. This, for instance, ensures that an intermediate produced by Strain A can be exported and taken up efficiently by Strain B, either by free diffusions or via membrane transporters. To increase flexibility in the design of metabolic modules, several studies explored (over-)expression of native or heterologous transporter genes, which in several cases had to be newly identified.

In a study aimed at metabolic engineering of *E. coli* for production of *cis,cis*-muconate, Zhang *et al.* [68] observed accumulation of the shikimate pathway intermediate

3-dehydroshikimate (DHS) by engineered strains. Based on this observation, a co-culture strategy was devised, in which a first *E. coli* strain converted xylose to DHS via the shikimate pathway, while a second strain, grown on glucose as carbon source, converted DHS to *cis,cis*-muconate via three heterologous enzymes. The inability of *E. coli* to import DHS under the experimental conditions was addressed by identification and overexpression, in the latter strain, of a native gene that encoded a functional DHS transporter. After optimization of strains and process conditions, this co-cultivation strategy enabled a *cis,cis*-muconate yield on sugar that corresponded to 51% of the theoretical maximum.

Transporter engineering with the aim to improve co-culture performance can also involve export of key intermediates, as exemplified by a study on co-cultures of two *E. coli* strains [69], of which the first overexpressed and exported tyrosine, which was then converted to 4-hydroxystyrene by a second strain expressing two heterologous enzymes. Expression of a *Petunia* gene encoding a plastid amino-acid transporter improved export of tyrosine by the former strain, thereby enabling a two-fold higher 4-hydroxystyrene titer in co-cultures than observed in a co-culture with a strain lacking the heterologous transporter.

A division-of-labour strategy for producing the benzyloquinoline alkaloid precursor (S)-norcoclaurine was based on the availability of a *Scheffersomyces stipitis* strain that produced high levels of the pathway intermediate shikimate [28]. Since genetic engineering tools for this non-conventional yeast were less well developed than for *S. cerevisiae*, a co-cultivation strategy was based on conversion of shikimate, produced by *S. stipitis*, to (S)-norcoclaurine by an extensively engineered *S. cerevisiae* strain. The inability of *S. cerevisiae* to efficiently import shikimate was addressed by identification of two *Aspergillus niger* shikimate transporter genes and their functional expression in the (S)-norcoclaurine-producing *S. cerevisiae* strain. This strategy enabled (S)-norcoclaurine titers in co-cultures that were two orders of magnitude higher than observed in a monoculture of the *S. cerevisiae* strain. Flexibility in modularization of the long biosynthetic pathways towards benzyloquinoline alkaloids was further extended by a study on identification, expression in *S. cerevisiae* and functional analysis of six *Papaver somniferum* benzyloquinoline alkaloid transporter genes [70]. Using these transporters, the opiate pathway was split into three modules assigned to three *S. cerevisiae* strains. Engineering of the strain carrying the ultimate reactions enabled formation of different alkaloid products via a mix and match strategy.

Identification and functional expression of relevant transporters increases options to further refine optimization of modular co-cultivation strategies, for example to prevent uneven distribution of pathway proteins over co-cultivated strains, based on algorithms for calculating protein allocation such as Gecko [71,72]. In addition, it

may be possible to use energy-coupled transport mechanisms to optimize substrate saturation of low- $k_{\text{cat}}$  enzymes and/or thermodynamic driving force for in vivo activity of spatially segregated metabolic models. The latter concept was theoretically explored by Bekiaris *et al.* [58], who developed the ASTHERISC algorithm. This algorithm integrates genome-scale models with thermodynamic reaction parameters to predict optimal pathway module definition. Although such options to integrate systems biology into the design of co-cultivation strategies are highly interesting, we are not aware of studies in which they have been experimentally tested.

### 1.3.3 Optimizing and controlling relative abundance of co-cultivation partners.

Achieving and maintaining an optimal relative abundance of co-culture partners is a key challenge in division-of-labour strategies that are based on spatial segregation of product-pathway modules. This is important, because imbalance between the co-culture partners can lead to one outperforming the other, resulting in suboptimal production. Not surprisingly, modifying inoculum ratios is a common and often successful approach for optimizing the performance of such co-cultures in laboratory studies (see e.g. [22,25,73,74]). However, specific growth rates of individual strains, and impacts of changing conditions during growth, can lead to population dynamics that negatively affect co-culture performance. In the absence of measures to stabilize relative abundance, impacts of population dynamics are likely to be even more pronounced under the intensive, dynamic cultivation conditions in large-scale industrial processes. Co-culture systems might therefore require monitoring of cell concentrations and the use of feedback control to maintain the balance. This can, for example, be done by process analytical techniques, as discussed in a recent review [75].

The use of mixtures of carbon substrates, of which only one can be used by each strain, has been successfully applied to stabilize relative abundance of strains in co-cultures (e.g. [68,76,77]). Alternatively, using consortium partners with different auxotrophic requirements enables the control of their relative abundance by supplying the required growth factors. This concept was used by Treloar *et al.* [78] to develop a deep-reinforcement-learning strategy to control relative abundance of two *E. coli* strains of which one was auxotrophic for arginine and the other for tryptophan. Simulations demonstrated the potential of this approach to control population composition and optimize productivity when supply of strain-specific substrates or growth factors can be coupled to online analysis of product concentration, for example by Raman spectroscopy or reporter proteins [79,80].

Inspired by studies in microbial ecology [81], self-stabilization of population composition can be based on mutual complementation of auxotrophies by

cross-feeding of growth factors [74,82,83]. Li *et al.* [84] explored how introducing such mutual dependencies can be applied to stabilize co-cultures of an *E. coli* strain that produces caffeate with a second strain that converts caffeate to salidroside. Strains expressing one of the two pathway modules were engineered to depend on production of either glutamate-derived amino acids or TCA-cycle intermediates by the other strain. In batch co-cultures of the mutually dependent strains, salidroside titers measured at the end of fermentation were independent of the inoculation ratio and over 2-fold higher than in cultures grown without engineered auxotrophies. The same study also demonstrated how, in the same context, a synthetic regulatory circuit based on a caffeate-responsive biosensor was used to enable ‘on-demand’ modulation of culture composition. To this end, the biosensor was coupled to expression of a degradation-prone version of a key enzyme in glutamate metabolism, thereby tuning the abundance of the caffeate-producing strain to its consumption by the salidroside-producing strain [84].

While the approaches discussed above demonstrate how relative abundance of strains in laboratory co-cultures can be stabilized and even be subjected to ‘on-demand tuning’, we have not found studies in which genetic stability of such advanced co-cultures has been tested during long-term cultivation.

## 1.4 Outlook

There are few documented examples of the large-scale application of defined microbial co-cultures for production of low-molecular-weight compounds [9,85]. However, these reports either contain limited information [9], or are a bioconversion type of process [85]. This is not surprising as, from an industrial perspective, development and implementation of co-culture-based processes introduces additional levels of complexity compared to mono-culture-based processes. Inspired by rapid developments in synthetic biology and protein engineering [86,87], industrial research may therefore, especially for issues such as pathway imbalance, preferentially explore options for advanced engineering of monocultures. In this outlook, we provide suggestions on how future academic research may help to more precisely define situations in which defined co-cultures can, in an industrial context, outperform mono-cultures.

### 1.4.1 Start with the end in mind

When design, construction and analysis of co-cultures are performed with the aim to contribute to development of industrial processes, they should, just like other research in bioprocess engineering [88], start with the intended industrial application firmly in mind. The design of co-culture strategies should be based on a clear definition of which industrially relevant challenges in monocultures are addressed. Carefully designed experiments will be needed to evaluate co-culture performance

under industrial conditions - for example, controlling starting strain ratios, feeding strategies, and using new bioreactor setups that can accommodate multiple organisms. This problem-based approach is illustrated by studies on the use of co-cultures for circumventing enzyme promiscuity [23,24], non-compatibility of the expression of specific enzymes with production strains [27,65] and genetic instability in generalist strains growing on sugar mixtures [21]. In contrast, problem definition is often less explicit when use of co-cultures is motivated from redistribution of metabolic burden, sometimes based on the incorrect assumption that equal distribution of product-pathway protein over co-culture partners will by definition lead to improved biomass-specific or volumetric productivity [57]. Moving beyond such intuitive design will benefit from rigorous quantitative comparison of mono- and co-cultures by integration of theoretical and experimental research on proteome allocation, bioenergetics and thermodynamics in mono- and co-cultures. Such systems biology approaches are also relevant for further optimizing the performance of co-cultures of substrate-specialists for production of dissimilatory products. In most of the academic studies cited in this review, co-culture performance was assessed based on product titer in small-scale laboratory batch cultures. We see a large potential for studies that, based on the existing and highly valuable body of knowledge, aim to quantitatively evaluate co-culture performance in terms of titer, productivity and yield [10] under simulated industrial conditions.

#### **1.4.2 Compare deterioration of product formation in mono- and co-cultures**

In processes aimed at dissimilatory product formation from mixed carbon substrates, co-cultures of 'carbon substrate specialists' have a clear potential to prevent the deterioration of fermentation kinetics observed in monocultures of generalist strains [21]. The industrial relevance of this co-cultivation strategy will, in practice, depend on whether strain robustness and feedstock composition allow for biomass recycling through multiple subsequent fermentation cycles. In contrast to dissimilatory product formation, synthesis of assimilatory products competes with growth for cellular energy, proteome space and biosynthetic precursors. This competition provides a selective advantage to mutants with reduced productivity. The impact of such 'protein burden' effects on the genetic stability of co-cultures for assimilatory product formation has not yet been studied in detail. Addressing this knowledge gap is of paramount importance for assessing and improving applicability in industrial processes that, from frozen stock culture to full scale, involve many generations of growth [89].

#### **4.3 Control the relative abundance of the strains**

Controlling the relative abundance of co-culture partners under dynamic industrial process conditions can be challenging and requires robust strategies for population

control. Techniques such as metabolic engineering of auxotrophic dependencies [74] and model-driven feedback control of key process parameters [78,90] are emerging solutions, but their performance requires validation under industrially relevant conditions. Further development of modeling tools (like in [91,92]) and improving techniques for monitoring and controlling population composition [75] will be crucial to unlocking the potential for industrial application.

We hope that, by inspiring readers to engage with the challenges involved in defined microbial co-cultures, this paper will contribute to the development of these scientifically fascinating systems into mature production platforms for microbial biotechnology. With ongoing advances in synthetic biology and bioprocess engineering, ever more tools become available to fine-tune interactions between co-culture partners and, thereby, optimize stable performance. In addition, quantitative analysis and optimization of co-cultures under simulated industrial conditions is essential for understanding and improving performance of co-cultures in large-scale processes. We are convinced that such research will contribute to a paradigm shift in industrial technology that will lead to much wider use of co-cultures.

## Conclusion

Defined co-cultures offer unique opportunities to address several problems associated with the use of monoculture processes for production of assimilatory and dissimilatory products. Examples discussed in this review demonstrate, in laboratory-scale experiments, the use of well-designed co-cultivation strategies can improve yields, expand substrate ranges, prevent genetic instability, and mitigate pathway imbalances.

The next challenge is to gear research towards transitioning from laboratory-scale experiments, often with product titer as main read-out, to full-scale industrial processes. Integration of quantitative studies on co-culture performance under simulated, dynamic industrial process conditions with synthetic biology approaches for optimizing co-culture performance offers an excellent perspective to achieve this goal.

## Author Contributions

SBG: Investigation, Writing – Original Draft. TF: Investigation, Writing – Original draft. MR: Writing – Review & Editing, Visualization. JTP: Conceptualization, Writing – Original draft, Writing – Review & Editing, Supervision. RJvT: Conceptualization, Writing – Review & Editing, Supervision.

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# Scope of this Thesis

The general scope of this thesis is to investigate ecological principles relevant to defined co-cultures, identify biotechnological areas in which they can contribute to more efficient bioprocesses, and explore strategies for their industrial application.

**Chapter 1** discusses the potential applications of defined co-cultures both for assimilatory and dissimilatory product formation, and challenges associated with their industrial application.

**Chapter 2** proposes and assesses a strategy for increasing volumetric productivity in sequential batch cultures of defined co-cultures of sugar-specialist strains. In these conditions, generalist strains that can consume all carbon sources are often genetically unstable. The use of defined co-cultures of specialist strains may alleviate this problem but suffers from inefficiencies introduced by a disbalance in consumption rate by the consortium members. After assessing the significance of this inefficiency, experiments are presented that explore if selective sedimentation of a slow-growing consortium member may be a viable countermeasure.

**Chapter 3** explores ecological principles in spatially defined co-cultures. Using computational modeling and experimental approaches, parameters are investigated that determine the success of cooperators when facing a cheater population in a three-member consortium of bacteria.

**Chapter 4** investigates limitations of microbial droplet screening techniques that have gained popularity in recent years for screening individual strains, as well as for co-culture studies. These setups often consist of aqueous droplets dispersed in an oil phase, which compartmentalizes the cells and molecules present in the droplets. Based on experiments with biosensors, the chapter assesses how well this compartmentalization holds for different biologically relevant molecules.





*Everything that rises must be yeast.*

TC Boyle, *Water Music* (1981)

*Rise's Law* from *Ned Rise*, protagonist

## Chapter 2

# **Sedimentation accelerates mixed-substrate conversion during sequential batch cultivation of a co-culture of sugar-specialist yeast strains**

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Essentially as published in Applied and Environmental Microbiology (2026), 0:e00516-26, 1-17 <https://doi.org/10.1128/aem.00516-026>

## Abstract

Current industrial processes for yeast-based conversion of glucose, xylose, and arabinose mixtures in lignocellulosic hydrolysates mostly rely on monocultures of generalist strains that consume the sugars sequentially. Co-cultivation of 'sugar-specialist' strains offers potential advantages regarding volumetric productivity and genetic stability of pentose-fermenting strains during prolonged operation. However, in these cultures, the slow fermentation of pentoses strongly affects the time required for complete sugar conversion. This study explores if the kinetics of such co-cultures can be improved by increasing the biomass concentration through selective sedimentation of a slow-fermenting strain in sequential batch fermentations. Deletion of *ACE2* in a diploid, arabinose-specialist *Saccharomyces cerevisiae* strain yielded a multicellular, fast-sedimenting phenotype. In monocultures on 20 g·L<sup>-1</sup> arabinose in anaerobic sequential batch bioreactors with a 30-min biomass settling period between cultivation cycles, the sedimenting strain showed a shorter time for full conversion than its non-sedimenting parental strain ( $11.5 \pm 0.4$  h and  $16.6 \pm 0.4$  h, respectively). Co-cultures of a glucose-specialist *S. cerevisiae* strain with the sedimenting arabinose specialist on a glucose-arabinose mixture (10 g·L<sup>-1</sup> of each sugar) showed a 29% shorter batch time than corresponding co-cultures with the non-sedimenting arabinose-specialist ( $12.6 \pm 0.1$  h vs.  $17.7 \pm 0.1$  h). Increasing the biomass settling period to 1 h further reduced the batch time to  $8.1 \pm 0.1$  h and resulted in near-simultaneous depletion of both sugars. These results show that, if selective sedimentation of specific slow-fermenting strains can be implemented under relevant process conditions, it has the potential to improve microbial conversion of mixed substrates.

## 2.1 Introduction

Current large-scale processes in industrial biotechnology, including production of ethanol with the yeast *Saccharomyces cerevisiae*, mainly rely on the conversion of ‘first-generation’ feedstocks like hydrolysed corn starch or cane sugar [1,2]. Over the past decades, use of lignocellulosic biomass hydrolysates, derived from agricultural residues or energy crops, have received increased attention as a means to reduce land use and carbon emissions associated with ethanol production [1,3]. Unlike the glucose-rich hydrolysate obtained from corn starch or sugarcane, these second-generation feedstocks contain a mixture of the hexose glucose, and the pentoses D-xylose and L-arabinose (henceforth xylose and arabinose) as major fermentable sugars [4].

To maximize productivity and product yield on the carbon source, all three sugars must be fermented rapidly and completely. Yet, native *S. cerevisiae* strains cannot ferment xylose and arabinose [3,5]. Intensive research efforts have successfully identified and engineered heterologous metabolic pathways that can be inserted into *S. cerevisiae*, enabling the organism to ferment all three lignocellulosic sugars in anaerobic batch cultures [4,6]. However, the fermentation kinetics of such ‘generalist’ strains are often characterized by sequential sugar consumption [6,7]. These bi-phasic fermentation kinetics have been attributed to lower biomass-specific fermentation rates for the pentose sugars, glucose-repression mechanisms and competitive inhibition of pentose uptake by glucose [5,8]. Delayed pentose consumption, which can be exacerbated by a diauxic adaptation phase negatively affects volumetric productivity [9,10].

An additional drawback of the use of generalist strains emerged in experiments with sequential batch cultures, in which each subsequent batch cultivation cycle is initiated with a culture sample from the previous cycle. In ‘single sugar’ media, sequential batch cultivation can be used to increase volumetric productivity and even confers a selective advantage to fast fermenting mutants [6,11]. In contrast, prolonged sequential batch cultivation of generalist strains on mixtures of glucose and pentose sugars resulted in a progressive increase of the time required for complete sugar conversion, caused by deterioration of fermentation kinetics for both pentoses [7,12].

To overcome the limitations of pathway degeneration and sequential consumption, previous studies constructed co-cultures of specialist *S. cerevisiae* strains, each consuming only a single sugar [7,13]. In these studies, a wildtype *S. cerevisiae* strain, able to consume only glucose, was cultured with other strains, engineered and evolved to only ferment the pentoses xylose or arabinose. Although this strategy prevented pathway degeneration and ensured parallel consumption of all sugars, it

introduced new challenges related to the growth kinetics.

In a typical bioprocess, the consumption rate ( $R_s$ ,  $\text{mmol} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ ) of a strain for a sugar is the product of its biomass concentration ( $C_x$ ,  $\text{g} \cdot \text{L}^{-1}$ ) and its biomass specific consumption rate ( $q_s$ ,  $\text{mmol} \cdot \text{g}_{\text{Biomass}}^{-1} \cdot \text{h}^{-1}$ ) for this sugar ( $R_s = C_x \cdot q_s$ ) [14]. Pentose specialist strains often have a lower biomass specific consumption rate than glucose specialists, due to transport and regulation constraints, resulting in slow growth and consumption ( $R_s$ ) throughout the process [5,15,16]. This results in biphasic growth dynamics, where the fast specialist consumes its sugar to depletion and then remains idle until the slower strains consumed their designated sugar. On the other hand, because a generalist strain consumes the glucose first, it starts the pentose consumption phase with a higher biomass concentration ( $C_x$ ), augmenting subsequent pentose consumption rates and overall productivity [7]. Increasing the initial inoculum size of the slower growing pentose specialists can mitigate this issue in batch cultures, but the solution is constrained by the total available cell mass for inoculation generated in the seed train [7,17,18].

Repeated or semi-continuous operation modes present an opportunity for optimizing the fermentation kinetics of sugar specialist strains in consortia. These operation modes are characterized by periodic removal of spent medium, often retaining the biomass, as is done extensively in the Brazilian bioethanol industry [19]. Directly modifying relative inoculum sizes to enhance pentose fermentation rate is not possible in sequential batch co-cultures when each new cycle is inoculated with a representative sample of a previous, ideally mixed co-culture. However, one potential strategy to enhance the overall kinetics in such a system is to selectively increase the pentose specialists' biomass concentration across the batch cycles through selective biomass retention – preferentially maintaining the biomass of the strain during a biomass-settling phase [14,20]. Selective retention would provide the slow-growing strain with a competitive advantage by starting the subsequent batch with a higher inoculum and thereby accelerating pentose consumption.

To achieve selective retention, the slow-growing strain must sediment more rapidly than the fast-growing strain in a co-culture. One way to selectively induce aggregation and sedimentation in *S. cerevisiae* cells involves deletion of the *ACE2* gene [21]. *ACE2* encodes a transcriptional regulator that controls the expression of several genes involved in the final stage of cell cycle, including the chitinase *CTS1* required for mother-daughter cell separation after budding [22]. Inactivation of *ACE2* prevents separation of the budding cell from the mother, producing multicellular clonal aggregates [21,23]. Due to the different bud-localization preference in haploid and diploid *S. cerevisiae* cells, this effect is especially pronounced in diploid *ace2Δ ace2Δ* mutants, in which multicellular aggregates can reach diameters of hundreds of micrometres in size. These aggregates sediment rapidly and were previously shown

to cause selective biomass retention in sequential batch cultures [21,24].

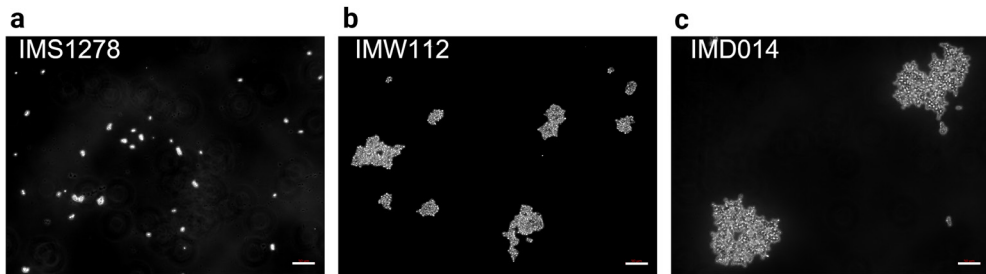
In this study, we investigate whether selective sedimentation of the slowest growing strain in a consortium of sugar-specialist yeast strains can improve volumetric productivity during sequential batch bioreactor cultivation on sugar mixtures. We first engineered a diploid arabinose-specialist *S. cerevisiae* for rapid sedimentation by deleting both copies of *ACE2*. We then compared fermentation kinetics of this strain with those of the parental, non-sedimenting strain during growth in sequential batch fermentation on arabinose alone. Finally, we tested the impact of the sedimentation phenotype of the arabinose-specialist strain on fermentation kinetics in sequential batch cultures of a consortium with a glucose specialist strain on a glucose-arabinose mixture.

## 2.2 Results

### 2.2.1 Deletion of *ACE2* yields sedimenting, multi-cellular clusters in a diploid arabinose-consuming *S. cerevisiae* strain.

*S. cerevisiae* IMS1278, obtained by a combination of target genetic modification and laboratory evolution, cannot consume glucose or xylose but was previously shown to consume arabinose in co-cultures with glucose- and xylose specialist strains on a mixture of these three sugars [7]. In this co-culture, the arabinose specialist consumed arabinose slower than the glucose strain consumed glucose [7]. To mitigate the consumption difference in the co-culture, we wanted to construct and employ a sedimenting arabinose strain based on IMS1278.

We based our strategy to construct a fast-sedimenting arabinose-specialist strain on earlier work, which showed that inactivation of both copies of *ACE2* in diploid *S. cerevisiae* strains causes a multi-cellular, fast-sedimenting phenotype [21]. Analysis of the genome sequence of IMS1278 with the MAGNOLYA algorithm [25] indicated that it had undergone whole-genome duplication (Supplementary Figure S2.1). We confirmed the diploidy by flow-cytometry analysis of Sytox Green-stained suspensions, using *S. cerevisiae* strains with known ploidies as references (Supplementary Figure S2.2). Inactivation of both copies of *ACE2* in strain IMS1278 by Cas9-assisted genome editing yielded *S. cerevisiae* IMW112. In contrast to its parental strain IMS1278 (Figure 2.1a), which showed a single-cell morphology, strain IMW112 formed multicellular clusters when grown in aerobic shake-flask cultures on synthetic medium with 20 g · L<sup>-1</sup> arabinose (SMA) (Figure 2.1b). The diameter of these clusters was, however, smaller than those observed in cultures of the previously constructed, pentose-non-fermenting strain IMD014 grown on synthetic medium with 20 g · L<sup>-1</sup> glucose (SMD) (Figure 2.1c, [21]).

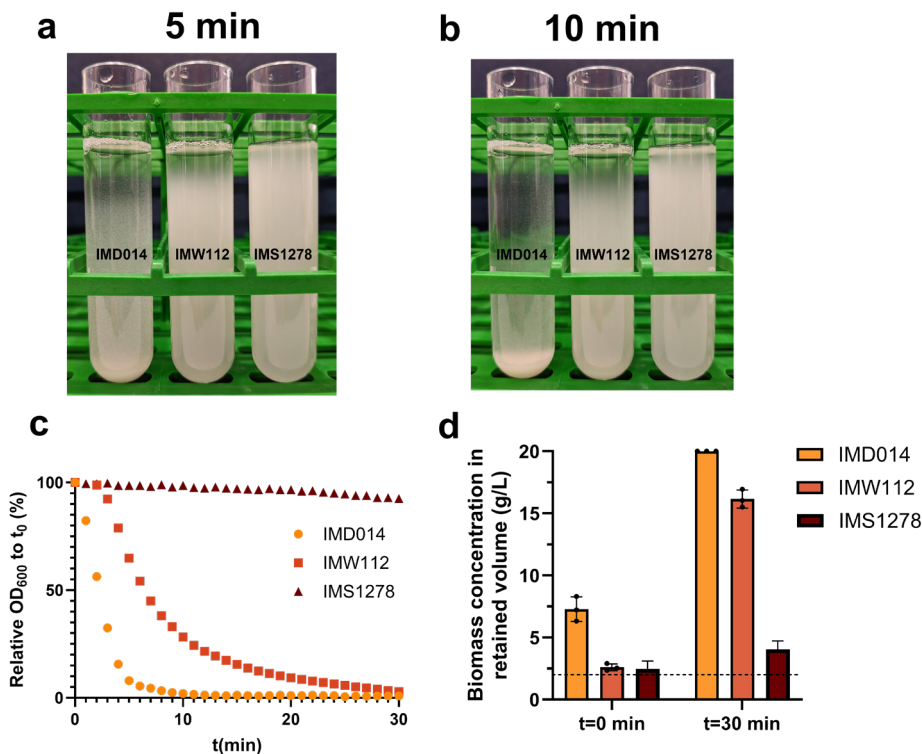


**Figure 2.1: Occurrence of multi-cellular morphology in *S. cerevisiae* strains.** a) *S. cerevisiae* IMS1278; diploid *ACE2/ACE2* arabinose-specialist strain (see Table 2 for genotype description), grown in aerobic shake-flask culture on SM-arabinose (SMA). b) *S. cerevisiae* IMW112;  $\Delta ace2/\Delta ace2$  mutant of strain IMS1278, grown in aerobic shake-flask culture on SM + arabinose (SMA); c) *S. cerevisiae* IMD014  $\Delta ace2/\Delta ace2$  pentose-non-fermenting reference strain, grown on SM + glucose (SMD). Samples were taken at stationary phase, marker bar indicates 50  $\mu\text{m}$ .

To test whether the multicellular morphology of strain IMW112 led to fast sedimentation, briefly vortexed cell suspensions of strains IMW112, IMS1278, and IMD014 were incubated in stationary glass tubes. After 5 and 10 min, clear zones appeared at the top of suspensions of each of the two  $\Delta ace2/\Delta ace2$  strains, while no such zones were observed in suspensions of the *ACE2/ACE2* strain IMS1278 (Figure 2.2a+b). These results showed that, consistent with its smaller cell-cluster size (Figure 2.1), strain IMW112 sedimented slower than the pentose-non-fermenting  $\Delta ace2/\Delta ace2$  strain. A further comparison of the sedimentation dynamics of these three strains confirmed this interpretation. When pre-mixed cell suspensions were statically incubated in a spectrophotometer cuvette, strain IMW112 reached 25 % of the initial  $\text{OD}_{660}$  after 10 min, while strain IMD014 already reached this reduction after 5 min (Figure 2.2c).

To investigate if the sedimentation rate of strain IMW112 would be sufficient to support cell retention in a sequential-batch bioreactor set-up with a 10-min emptying phase, a simulation experiment was performed in 15-mL tubes. We added 10 mL culture to the tube and removed 1 mL from the 1 mL (10 % volume) mark at 1-min intervals, which corresponds to the medium removal in a bioreactor. Subsequently, we assessed the biomass concentration in the remaining 1 mL by measuring the biomass in the removed fraction and subtracting it from the total biomass. When no prior static incubation step was performed, the biomass in the remaining suspension had increased by 3.5-fold for the IMD014 strain, but only by 1.3-fold in the IMW112 strain and remained at the initial concentration in the *ACE2/ACE2* strain IMS1278 (Figure 2.2d). After implementing a 30-min static incubation phase prior before initiating the emptying steps, the residual biomass concentration in the remaining 1-mL sample of suspensions of strain IMW112 was 8-fold higher than the initial biomass concentration. When subjected to the same procedure, strain IMS1278

showed an approximately 2-fold higher biomass concentration in the final sample (Figure 2.2d). Based on these observations, a settling time of 30 min was chosen for sequential batch bioreactor experiments.



**Figure 2.2: Sedimentation in suspensions of diploid *S. cerevisiae* strains** IMD014 ( $\Delta ace2/\Delta ace2$  pentose-non-fermenting reference strain), IMW112 ( $\Delta ace2/\Delta ace2$  mutant of arabinose specialist strain IMS1278, see Table 2.2 for genotype description), and IMS1278 ( $ACE2/ACE2$  arabinose-specialist strain). a) Pre-mixed cell suspensions after 5 min of static incubation; b) Pre-mixed cell suspensions after 10 min of static incubation; c) Monitoring of optical density at 660nm (OD<sub>660</sub>) during static incubation of cell suspensions in a spectrophotometer. d) Simulation, in 15-mL glass tubes of biomass sedimentation during the 10-min emptying phase of a sequential batch bioreactor. After adding 10 mL of a premixed 2.0 g biomass · L<sup>-1</sup> cell suspension, 1 mL-samples were removed at 1-min intervals from a point corresponding with a residual volume of 1 mL. The biomass concentration in the remaining 1 mL suspension was then measured. Experiments were performed with and without a prior 30 min settling period. Data represent average ± SD of three replicate experiments for each strain. Dotted line represents the starting concentration of 2.0 g biomass · L<sup>-1</sup>. Strains were pre-grown as indicated in the legend of Figure 2.1.

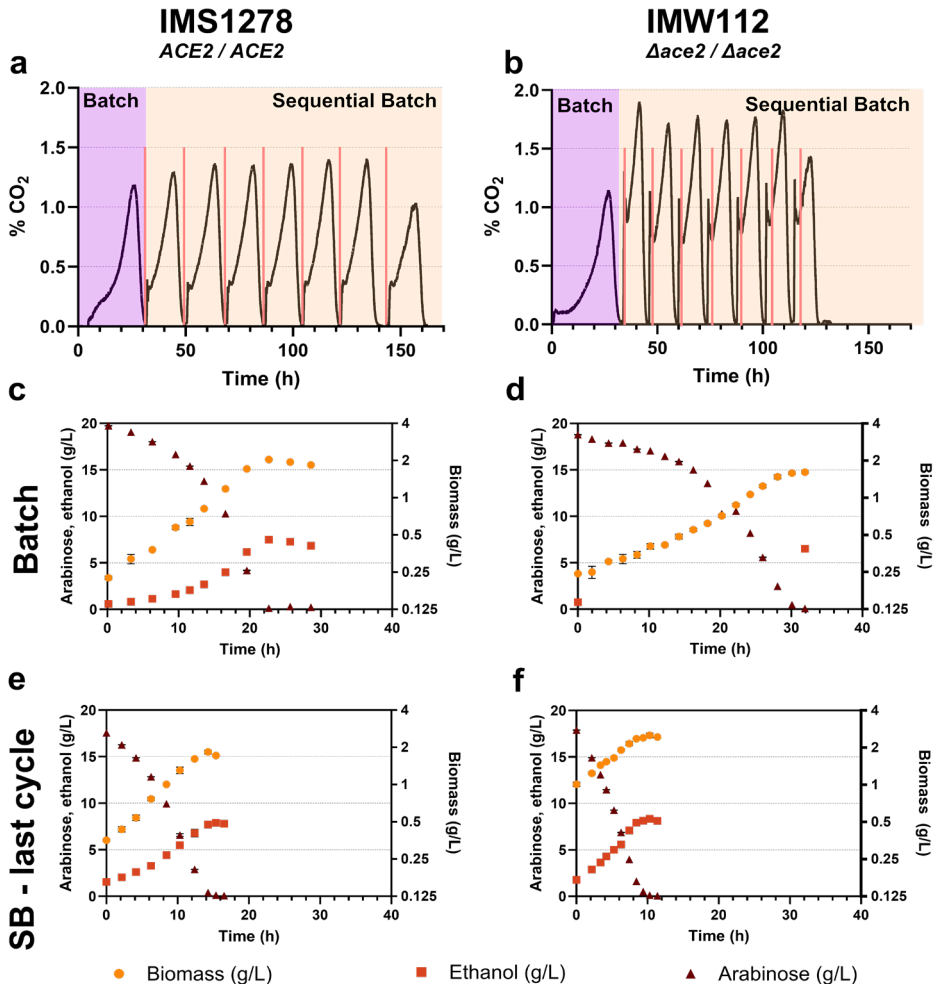
## 2.2.2 Biomass retention and arabinose conversion kinetics in sequential-batch monocultures

2 To compare biomass retention and arabinose conversion kinetics of the arabinose-specialist strains IMS1278 (*ACE2/ACE2*) and IMW112 ( $\Delta ace2/\Delta ace2$ ) in anaerobic sequential batch cultures on SMA, they were first grown as monocultures. In the initial bioreactor batch cultures, strain IMW112 grew slower than the strain IMS1278 (Figure 2.3a+b, IMW112:  $\mu_{\max}=0.080\text{ h}^{-1}$ ;  $\mu_{\max}=0.099\text{ h}^{-1}$ , respectively, Table 2.1). The time required to achieve full arabinose depletion ( $p\text{CO}_2$  in off-gas  $< 0.05\%$ ) for strain IMS1278 was  $24.1 \pm 1.6\text{ h}$ , as compared to  $31.5 \pm 0.5\text{ h}$  for the strain IMW112 (Figure 2.3b). The ethanol yield was similar for both strains (Table 2.1).

To investigate the impact of biomass retention of the sedimenting  $\Delta ace2/\Delta ace2$  strain IMW112 on fermentation kinetics in sequential-batch cultures, we compared its performance in such cultures with that of the non-sedimenting *ACE2/ACE2* strain IMS1278. The sequential-batch bioreactors were programmed to automatically switch off the stirrer and aeration when a decrease of the  $\text{CO}_2$  concentration below a preset value indicated arabinose depletion. After a 30-min settling time, 90% of the culture volume was then automatically replaced by fresh medium (see methods section).

Except for a difference in specific growth rate, the two strains showed similar off-gas  $\text{CO}_2$  profiles in the batch culture that preceded the sequential-batch mode (Figure 2.3a). After this, the non-clustering *ACE2/ACE2* strain IMS1278 maintained the same profile in each subsequent cycle, and the  $\Delta ace2/\Delta ace2$  strain IMW112 started each subsequent cycle with a higher initial off-gas  $\text{CO}_2$  concentration, which was indicative for biomass retention (Figure 2.3a). In addition, during sequential-batch operation with strain IMW112, the duration of each cycle was shorter than the length of the initial batch phase (Figure 2.3a). Biomass and metabolite analyses in the seventh cycle of sequential-batch operation confirmed the impact of sedimentation. Reactors with strain IMW112 initiated this cycle with  $0.97 \pm 0.06\text{ g} \cdot \text{L}^{-1}$  biomass, resulting in arabinose depletion ( $p\text{CO}_2$  in off-gas  $< 0.05\%$ ) in  $11.3 \pm 0.4\text{ h}$ . This time was 32 % shorter than observed in reactors with strain IMS1278, which started at  $0.33 \pm 0.03\text{ g} \cdot \text{L}^{-1}$  biomass and required  $16.6 \pm 0.4\text{ h}$  for arabinose depletion (Figure 2.3c). Both strains show a higher specific growth rate in the seventh cycle of sequential-batch operation than in the initial anaerobic batch cultures, which may indicate an adaptation to the anaerobic conditions (Table 2.1).

The results show that accelerated sedimentation of strain IMW112 resulted in biomass retention in sequential batch cultures, thereby enabling higher volumetric conversion rates than observed with a reference strain.



**Figure 2.3: Cluster formation increases biomass retention and arabinose consumption across cycles in a sequential batch setup.** Fermentation kinetics of diploid *S. cerevisiae* strains IMS1278 (Panels a,c,e; *ACE2/ACE2* arabinose-specialist strain, see Table 2.2 for genotype description) and IMW112 (Panels b,d,f; *Δace2/Δace2* mutant of strain IMS1278), grown in anaerobic sequential-batch bioreactors on SM with 20 g · L<sup>-1</sup> arabinose. a+b) CO<sub>2</sub> content of bioreactor off-gas during the initial batch culture and seven subsequent sequential batch cycles. Off-gas data for the final cycle are influenced by sampling for metabolite analyses. The 30-min settling phase is indicated in red. c+d) Concentrations of arabinose, biomass and ethanol in the initial anaerobic batch cultures of each strain. Cultures were inoculated with 0.2 g · L<sup>-1</sup> biomass. Due to an issue with the ethanol sampling procedure, only start and endpoint ethanol concentrations could be determined for the IMW112 strain. e+f) Concentrations of arabinose, biomass and ethanol in the seventh cycle of sequential-batch operation of each strain. Metabolite and biomass data shown in the figure represent average ± SD of technical replicates. Duplicate bioreactors were run for each strain, with data from one reactor shown in the figure; data for the other reactors are shown in Supplementary Figure S2.3.

**Table 2.1: Maximum specific growth rate ( $\mu_{\max}$ ), biomass yield, and ethanol yield of diploid *S. cerevisiae* strains IMS1278 (*ACE2/ACE2* arabinose-specialist strain; see Table 2.2 for genotype description) and IMW112 (*ace2/ $\Delta$ ace2* mutant of strain IMS1278), grown in anaerobic sequential batch bioreactors on SM with  $20 \text{ g} \cdot \text{L}^{-1}$  arabinose. Data are presented for initial anaerobic batch cultures and the last of seven subsequent sequential-batch cycles of each strain. Data represent average  $\pm$  SD of duplicate reactors for each strain.**

|                        | $\mu_{\max} \text{ (h}^{-1}\text{)}$ | Biomass yield ( $\text{g}_s/\text{g}_s$ ) | Ethanol yield ( $\text{g}_{\text{etoh}}/\text{g}_s$ ) |
|------------------------|--------------------------------------|-------------------------------------------|-------------------------------------------------------|
| IMS1278 batch          | $0.099 \pm 0.008$                    | $0.088 \pm 0.002$                         | $0.36 \pm 0.01$                                       |
| IMS1278 SBR last cycle | $0.14 \pm 0.01$                      | $0.083 \pm 0.000$                         | $0.36 \pm 0.00$                                       |
| IMW112 batch           | $0.080 \pm 0.002$                    | $0.078 \pm 0.003$                         | $0.35 \pm 0.00$                                       |
| IMW112 SBR last cycle  | $0.10 \pm 0.000$                     | $0.082 \pm 0.000$                         | $0.36 \pm 0.00$                                       |

### 2.2.3 Selective sedimentation of an arabinose specialist strain accelerates conversion of a glucose-arabinose mixture in sequential-batch co-cultures.

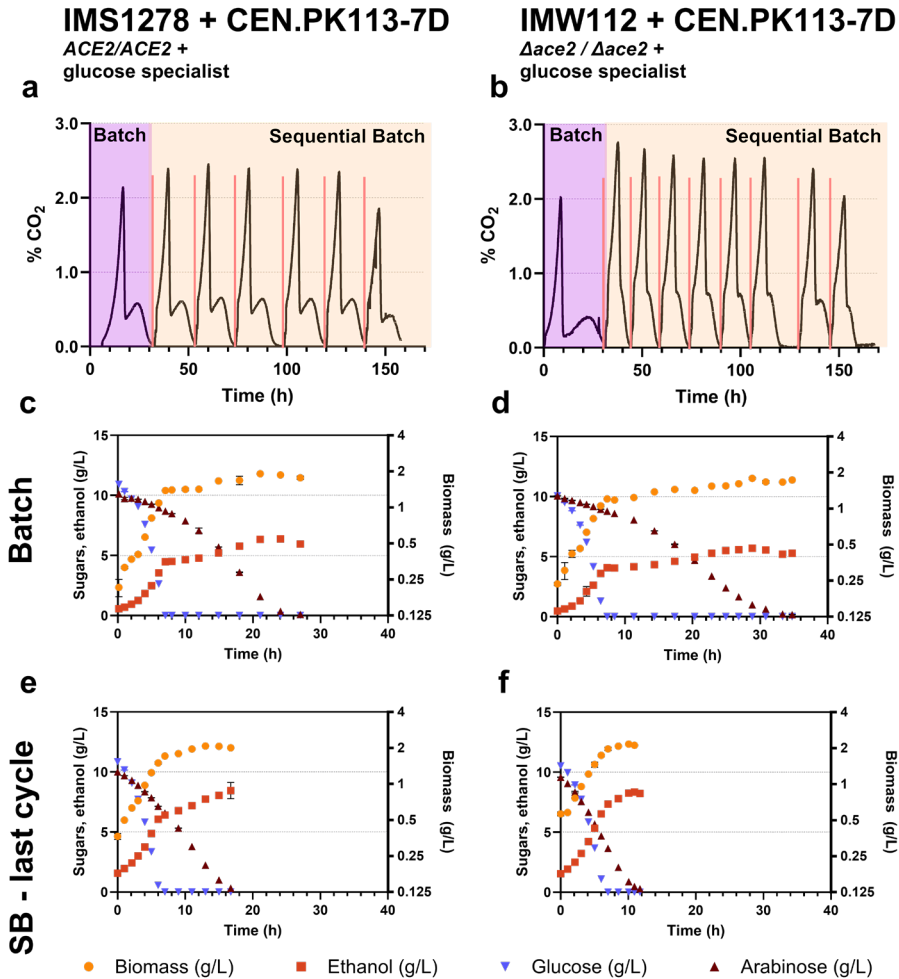
After demonstrating that sedimentation of the clustering strain IMW112 resulted in faster arabinose consumption after multiple sequential batch cycles, we next investigated the impact of selective sedimentation on the kinetics of mixed substrate conversion. We characterized the first and last cycle of an anaerobic sequential batch setup with  $10 \text{ g} \cdot \text{L}^{-1}$  each of arabinose and glucose, inoculated with  $0.1 \text{ g} \cdot \text{L}^{-1}$  CEN.PK113-7D and  $0.1 \text{ g} \cdot \text{L}^{-1}$  of either the non-clustering IMS1278 strain or the clustering IMW112 strain. By halving both the initial concentration of arabinose and the inoculation size of the arabinose specialist, we maintained the same biomass-to-substrate ratio as in the monoculture experiments. Therefore, we expected the time required for arabinose depletion to be similar to that observed for the monocultures.

The off-gas  $\text{CO}_2$  profile for the batch phase was similar for both strain combinations and showed two distinct peaks, suggesting two growth phases in the culture (Figure 2.4a). The biphasic  $\text{CO}_2$  profiles were consistent with complete glucose consumptions, in both co-cultures, within  $7.5 \text{ h} \pm 0.2 \text{ h}$ . Complete conversion of arabinose occurred within  $24.8 \pm 0.7 \text{ h}$  and  $30.1 \pm 0.1 \text{ h}$  for strains IMS1278 and IMW112, respectively (Figure 2.4b). Since the time to full conversion of arabinose was similar in co-culture as in monoculture, this suggests that the strains were unaffected by the presence of the glucose specialist. The faster consumption of arabinose by strain IMS1278 was consistent with its faster growth in anaerobic monocultures on arabinose (Figure 2.4).

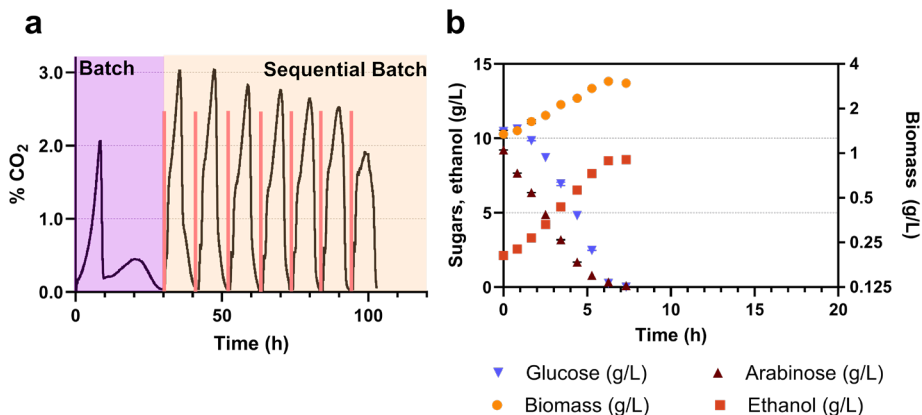
After the initial batch phase, the co-cultures were subjected to the same sequential-

batch regime as described above for monocultures of the arabinose specialist strains. Through six sequential-batch cycles, co-cultures of strain CEN.PK113-7D with the arabinose-specialist strain IMS1278 (*ACE2/ACE2*) maintained a CO<sub>2</sub> off-gas profile with two distinct peaks, while these distinct peaks were nearly absent in the co-culture with IMW112 (*Δace2/Δace2*) after the first cycle (Figure 2.4a). Analysis of the final cycle of these sequential batch cultures showed initial biomass concentrations of  $0.34 \pm 0.03 \text{ g} \cdot \text{L}^{-1}$  and  $0.56 \pm 0.00 \text{ g} \cdot \text{L}^{-1}$  for co-cultures with IMS1278 and IMW112, respectively (Figure 2.4c). In all four final cultures, glucose was consumed within  $7.8 \pm 0.3 \text{ h}$ . In these final cycles, co-cultures with strain IMW112 converted arabinose faster than strain IMS1278 ( $12.6 \pm 0.1 \text{ h}$  and  $17.7 \pm 0.1 \text{ h}$  required for arabinose conversion, respectively). This reduction by 29 % is consistent with its faster growth in anaerobic monocultures on arabinose (Figure 2.3).

To investigate if the positive impact of selective sedimentation of the arabinose-specialist strain IMW112 on arabinose conversion kinetics in sequential-batch co-cultures with the glucose specialist strain CEN.PK could be further increased, the settling time was increased from 30 min to 60 min. During sequential-batch cultivation, the off-gas CO<sub>2</sub> profile of these co-cultures showed only a single peak as opposed to the two distinct peaks in the first cycle, suggesting that both sugars were consumed simultaneously (Figure 2.5a). Measurements on the seventh cycle of duplicate cultures showed that in the initial biomass concentration had increased to  $1.36 \pm 0.02 \text{ g} \cdot \text{L}^{-1}$ , allowing the co-culture to simultaneously consume both sugars within  $8.1 \pm 0.1 \text{ h}$  (Figure 2.5b). These results show that the consumption rate of the sedimenting strain can be adjusted by changing the biomass settling time.



**Figure 2.4: Selective sedimentation of a cluster-forming arabinose-specialist strain accelerates conversion of a glucose-arabinose mixture in sequential batch co-cultures.** Fermentation kinetics of co-cultures of the pentose-non-fermenting reference strain ('glucose specialist') *S. cerevisiae* CEN.PK113-7D with either strain IMS1278 (panels a,c,e; *ACE2/ACE2* arabinose - specialist strain, see Table 2 for genotype description) or IMW112 (panels b,d,f; *Δace2/Δace2* mutant of strain IMS1278), grown in anaerobic sequential-batch bioreactors on SM with 10 g · L<sup>-1</sup> each of D-glucose and L-arabinose. a+b) CO<sub>2</sub> content of bioreactor off-gas during and initial batch culture and subsequent sequential-batch cycles. Off-gas data for the final cycles are influenced by sampling for metabolite analyses. The 30-min settling phase is indicated in red. c+d) Concentrations of arabinose, biomass and ethanol in the initial anaerobic batch cultures of each co-culture, inoculated with 0.1 g biomass · L<sup>-1</sup> of each strain. e+f) Concentrations of arabinose, biomass and ethanol in the final cycle of sequential-batch operation. Metabolite and biomass data shown in the figure represent average ± SD of technical replicates. Duplicate bioreactors were run for each strain combination, with data from one reactor shown in the figure; data for the other reactors are shown in Supplementary Figure S2.4.



**Figure 2.5: Extension of settling time results in simultaneous conversion of glucose and arabinose in a sequential batch co-culture.** Fermentation kinetics of co-cultures of the pentose-non-fermenting reference strain ('glucose specialist') *S. cerevisiae* CEN.PK113-7D and the arabinose-specialist strain IMW112 ( $\Delta ace2/\Delta ace2$ ) grown in anaerobic sequential-batch bioreactors on SM with  $10 \text{ g} \cdot \text{L}^{-1}$  each of glucose and arabinose. In comparison with the experiments shown in Figure 4, settling time after substrate depletion was extended from 30 to 60 min. a) CO<sub>2</sub> content of bioreactor off-gas during and initial batch culture and subsequent sequential-batch cycles. Off-gas data for the final cycle are influenced by sampling for metabolite analyses. The 60-min settling phase is indicated in red. b) Concentrations of arabinose, glucose, biomass, and ethanol in the final cycle of sequential-batch operation. Metabolite and biomass data shown in the figure represent average  $\pm$  SD of technical replicates. Duplicate bioreactors were run, with data from one reactor shown in the figure; data for the other reactor are shown in Supplementary Figure S2.5.

## 2.3 Discussion

In this study, we explored the application of sedimenting specialist *S. cerevisiae* strains to intensify mixed-substrate processes. Deletion of *ACE2* caused the strains to form multicellular clusters that settled more quickly than their unicellular progenitors. Increasing the relative biomass of the slower strain during repeated batch fermentation enabled fast and parallel consumption of all available carbon sources.

As previously shown in wild-type *S. cerevisiae* strain backgrounds [21,26], inactivation of *ACE2* in an engineered, diploid arabinose-specialist strain of *S. cerevisiae* conferred a multicellular, sedimenting phenotype (Figure 2.1, Figure 2.2). In anaerobic, arabinose-grown monocultures, the specific growth rate of the resulting strain was approximately 20% lower than that of its *ACE2/ACE2* parental strain (Table 2.1). The combination of a small size and low specific growth rate of the multicellular clusters (Figure 2.1), combined with the high ( $20 \text{ g} \cdot \text{L}^{-1}$ ) arabinose concentrations used in these cultures, rule out diffusion limitation as a plausible explanation for this

difference in growth rate. Instead, slower growth of the cluster-forming strain may reflect steric hindrance and/or mechanical stress experienced by cells in the clusters' cores [27]. Similarly, the small cluster size would likely not present a barrier for oxygen transfer to the center cells under aerobic conditions.

As per Stoke's law, the sedimentation velocity of a particle is proportional to the square of the cluster diameter. Arabinose-grown anaerobic cultures of the  $\Delta ace2/\Delta ace2$  arabinose-specialist strain showed smaller clusters and, consequently, slower sedimentation than glucose-grown cultures of a congenic  $\Delta ace2/\Delta ace2$  reference strain, (Figure 2.1, Figure 2.2). In clonal, multicellular clusters, size is determined by cluster age and its internal biophysical properties, particularly the maximal internal stress the cluster can withstand before fracture [28,29]. Previous studies have shown that the morphology of the constituent cells governs the cluster's stress tolerance [23,28,30]. The observed differences in cluster size between the two strains may therefore be caused by differences in cell size and aspect ratio [31]. Alternatively, it might reflect different residual expression levels of *CTS1*, which encodes the endochitinase involved in mother-daughter cell separation, in the absence of its regulator Ace2 [32]. Cell cluster size and settling rate of the sedimenting strain may be increased via evolutionary methods [33].

In anaerobic, arabinose-grown sequential-batch bioreactors, with a sedimentation time of 30 min between cycles, monocultures of the  $\Delta ace2/\Delta ace2$  arabinose-specialist showed clear biomass retention. This enabled shorter cycle times than in similar cultures of its congenic, non-sedimenting parental strain (Figure 2.3). Use of biomass sedimentation to enhance volumetric conversion rates in sequential batch cultures is, in itself, not a new concept. Granular-sludge systems are applied on a large scale in microbial waste-water treatment [34], flocculation is used for collection and subsequent repatching in brewing [35], and large-scale Brazilian bioethanol processes achieve up to 600 fermentation cycles per harvest season through the use of centrifugation to remove product and recycle biomass [14,19]. For the latter process, sedimentation via flocculation has been suggested as cost-saving alternative for the centrifugation step [36]. Bioreactor designs enabling the continuous removal of sedimenting cells in a binary culture have been explored previously and may be integrated in processes at larger scale [37]. We are not aware of prior studies that explore selective sedimentation of co-culture partners to balance their relative contributions to mixed-substrate conversion.

The present study shows that, in sequential-batch co-cultures of a fast-fermenting glucose-specialist strain and a much slower fermenting arabinose-specialist strain, cycle times for conversion of a glucose-arabinose mixture were strongly reduced by selective sedimentation of the latter during a short settling phase (Figure 2.4, Figure 2.5). This work may be extended to also include a xylose-specialist yeast

strain. If this concept can be applied for mixed-substrate conversion in large-scale processes, its advantages may extend beyond volumetric productivity. When, in larger cell clusters, specific growth rate can be significantly reduced, this may enhance yields on substrate of dissimilatory products such as ethanol by increasing relative contribution of maintenance energy metabolism to overall conversion rates [38, 39]. In addition, yeast-cell aggregates formed by flocculation have been shown to exhibit increased tolerance to inhibitors occurring in lignocellulosic hydrolysates [40].

We realize that the defined media and laboratory conditions employed in our study do not capture key challenges associated with feedstocks and bioreactor scales used in industry. Viscosity of the slurries obtained after hydrolysis of lignocellulosic feedstock is a known challenge for gravity-based biomass recycling, often addressed by integrating a liquid-solid separation step to lower viscosity [41, 42]. Application in such systems is likely to require further metabolic and/or evolutionary engineering to enhance biomass settling [33]. The simple approach described here may, however, be of immediate relevance in other co-culture contexts. For example, in division-of-labor strategies that distribute enzymes of long biosynthetic pathways for complex biomass components [43], co-culture partners that carry pathway enzymes with low *k<sub>cat</sub>* values may be specifically retained in sequential-batch cultures to maximize volumetric productivity.

This study was primarily inspired by industrial application of co-cultures. However, its results also illustrate how simple mutations that result in formation of multicellular clusters can confer a selective benefit to slow-growing microbial species and communities in micro-environments that naturally undergo ‘overflow’ events [44]. For yeasts such as *S. cerevisiae*, whose natural environments are associated with plants and trees [45], these might include environments subject to periodic washout by rainfall (e.g. tree bark or flower petals). Investigating if a sedimenting phenotype contributes to persistence provides an interesting avenue for ecological research.

## 2.4 Materials and Methods

### 2.4.1 Microbial strains and growth conditions

**Table 2.2: *S. cerevisiae* strains used in this study.**

| Strain name                          | Relevant Genotype                                                                                                                                                                                                                                                                                                                                                                                                                                                                  | Description                                                                                                                                                                                                                                                                           | Reference |
|--------------------------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| <i>S. cerevisiae</i><br>CEN.PK113-7D | <i>MATa MAL2-8c SUC2</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                           | Common laboratory strain (wildtype, 'glucose-specialist strain')                                                                                                                                                                                                                      | [46,47]   |
| <i>S. cerevisiae</i><br>IMS1278      | <i>MATa, ura3-52, his3-1, leu2-3112, MAL2-8c, SUC2, Δglk1::SpHIS5, Δhxx1::KILEU2, Δgal1::CAS9- AMDS, Δgre3::(pTDH3_RPE1, pPGK1_TKL1_tTKL1, pTEF1_TAL1_tTAL1, pPGI1_NQM1_tNQM1, pTPI1_RKI1_tRKI1, pPYK1_TKL2_tTKL2), Δgal80::(pTPI_LaaraA_tCYC)x9, pPYK_LaaraB_tPGI1, pPGK_LaaraD_tTDH3, Δhxx2::PcARAT</i><br><br><b>Genetic changes that occurred during evolution:</b><br>Whole-genome diploidization<br><i>GAL2</i> (N376I T89I),<br><i>BMH1</i> (E22*),<br><i>IPT1</i> (S313F), | Diploid arabinose-specialist strain that has undergone laboratory evolution on a mixture of arabinose, glucose, and xylose under anaerobic conditions first in monoculture, and then in co-culture with a glucose-, and a xylose specialist strain. All modifications are homozygous. | [7,48]    |
| <i>S. cerevisiae</i><br>IMW112       | IMS1278 <i>Δace2</i> / <i>Δace2</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                | Cluster-forming, fast-sedimenting diploid arabinose specialist strain generated by deleting both copies of <i>ACE2</i>                                                                                                                                                                | This work |
| <i>S. cerevisiae</i><br>IMD014       | <i>MATa/α ura3-52/URA3 ace2-2-loxP-HphNT1-loxP / ace2-2-loxP-KanMX-loxP</i>                                                                                                                                                                                                                                                                                                                                                                                                        | Diploid, cluster-forming, fast-sedimenting glucose specialist generated by mutating <i>ACE2</i> in both alleles.                                                                                                                                                                      | [21]      |
| <i>S. cerevisiae</i><br>CEN.PK122    | <i>MATa/MATα</i>                                                                                                                                                                                                                                                                                                                                                                                                                                                                   | Laboratory strain, diploid                                                                                                                                                                                                                                                            | [46,47]   |
| <i>S. cerevisiae</i><br>FRY153       | 3n                                                                                                                                                                                                                                                                                                                                                                                                                                                                                 | Triploid strain                                                                                                                                                                                                                                                                       | [49]      |

The *S. cerevisiae* strains used in this study (Table 2.2) were derived from the CEN.PK lineage, except for FRY153 [46,47]. Synthetic medium (SM), containing  $5.0 \text{ g} \cdot \text{L}^{-1} (\text{NH}_4)_2\text{SO}_4$ ,  $3.0 \text{ g} \cdot \text{L}^{-1} \text{KH}_2\text{PO}_4$ ,  $0.5 \text{ g} \cdot \text{L}^{-1} \text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , trace elements and vitamins was prepared as described previously [50]. Freezer stocks were prepared by growing the strains in SM with  $20 \text{ g} \cdot \text{L}^{-1}$  L-arabinose and storing the samples from exponentially growing cultures at  $-80^\circ\text{C}$  with 30 vol% glycerol. Yeast strains were pre-cultured in 100 mL SM with  $20 \text{ g} \cdot \text{L}^{-1}$  of the appropriate sugar in round-bottom 500 mL shake flasks in a shaking incubator at  $30^\circ\text{C}$ . Anaerobic growth medium was supplemented with ergosterol ( $10 \text{ mg} \cdot \text{L}^{-1}$ ) and Tween80 ( $420 \text{ mg} \cdot \text{L}^{-1}$ ) [51]. Chemically competent *Escherichia coli* XL1-Blue cells were cultured on Lysogeny Broth (LB), supplemented with  $100 \text{ mg} \cdot \text{L}^{-1}$  ampicillin (Merck, Darmstadt, Germany) where relevant. Solid media were prepared by adding  $20 \text{ g} \cdot \text{L}^{-1}$  agar (Becton Dickinson, Franklin Lakes, NJ) prior to heat sterilization. *E. coli* cultures on agar plates were incubated overnight at  $37^\circ\text{C}$  and *S. cerevisiae* plates were incubated at  $30^\circ\text{C}$  for 3 days.

### 2.4.2 Plasmid construction

A Cas9 gRNA target sequence in *ACE2* was identified as described previously [52]. To construct plasmid pUDR913 (Supplementary table S2.1) containing the gRNA, a linear backbone fragment of pROS13 was first PCR amplified with primer 6005 (Supplementary table S2.2) and the insert containing the gRNA was PCR-amplified with primer 20864 using Phusion DNA polymerase (Thermo Fisher). Backbone and insert were isolated from the agarose gel using the Zymoclean Gel DNA Recovery kit (Zymo Research, Irvine, CA). The plasmid was assembled using Gibson Assembly using a HiFi DNA Assembly master mix (New England Biolabs, Ipswich, MA). One  $\mu\text{L}$  of the mixture was used to transform *E. coli* XL1-Blue cells with a heat-shock protocol [53]. Plasmid pUDR913 was isolated from the transformed *E. coli* cells using the Sigma GenElute Plasmid Miniprep Kit (Sigma-Aldrich).

### 2.4.3 Gene editing

A dsDNA repair fragment for deletion of *ACE2* was prepared by mixing oligonucleotides 20865 and 20866 (Supplementary table S2.2) in a 1:1 molar ratio. The mixture was heated to  $95^\circ\text{C}$  for 5 min and cooled on ice. *S. cerevisiae* IMW112 was constructed by co-transforming strain IMS1278 with gRNA-plasmid pUDR913 (Supplementary table S2.1) and the *ACE2* repair fragment, using the lithium-acetate method [54]. Transformants were selected on SM plates supplemented with  $20 \text{ g} \cdot \text{L}^{-1}$  arabinose and  $200 \mu\text{g} \cdot \text{L}^{-1}$  hygromycin B. Correct deletion was checked by diagnostic PCR with DreamTaq polymerase (Thermo Fisher) using primers 20867 and 20868 (Supplementary table S2.2). pUDR913 was removed by growing transformants with confirmed genotype on non-selective SM supplemented with  $20 \text{ g} \cdot \text{L}^{-1}$  arabinose,

after which a cured IMW112 strain was stored at  $-80^{\circ}\text{C}$ .

#### 2.4.4 Ploidy analysis using flow cytometry

The ploidy of *S. cerevisiae* IMS1278 was determined using a flow cytometry protocol adapted from [55]. Cells were grown aerobically in SM +  $20\text{ g} \cdot \text{L}^{-1}$  arabinose for 24 h. In parallel, *S. cerevisiae* haploid, diploid and triploid (strains CEN.PK113-7D, CEN.PK122, and FRY153, respectively, Table 2.1) were grown overnight in SM +  $20\text{ g} \cdot \text{L}^{-1}$  glucose. After harvesting approximately  $10^7$  cells and washing once with demineralized  $\text{H}_2\text{O}$  (5 min, 6000 g), 1.6 mL 70 vol% ethanol was added while vortexing. After overnight incubation, the suspended cells were pelleted (4000 g, 4 min) and washed once with 500  $\mu\text{L}$  of 50 mM Tris-HCl (pH 7.5), after which cells were resuspended in 100  $\mu\text{L}$  of 50 mM Tris-HCl (pH 7.5) with  $1\text{ mg} \cdot \text{mL}^{-1}$  RNase A and incubated at  $37^{\circ}\text{C}$  for 2 h. Subsequently, 200  $\mu\text{L}$  of 50 mM Tris-HCl (pH 7.5) +  $7.5\text{ mg} \cdot \text{mL}^{-1}$  trypsin was added, the solution was vortexed and incubated at  $37^{\circ}\text{C}$  for 2 h. Afterwards, the cells were centrifuged and washed once with 200  $\mu\text{L}$  of 50 mM Tris-HCl (pH 7.5) and resuspended in 500  $\mu\text{L}$  of 50 mM Tris-HCl (pH 7.5). Per sample, 1 mL of 50 mM Tris-HCl (pH 7.5) + 1  $\mu\text{M}$  Sytox Green was prepared in dark Eppendorf tubes and 100  $\mu\text{L}$  sample was added, vortexed, and stored on ice. Cells were sonicated at 30 % (6  $\mu\text{m}$ ) for 15 s using a Soniprep 150 (Medical and Scientific Equipment, Nuaille, France). An Accuri C6 flow cytometer (Becton Dickinson, Franklin Lakes, NJ) was used to derive the distribution of DNA content per sample.

#### 2.4.5 Microscopy

Strains were grown to stationary phase in SM supplemented with the appropriate carbon source and imaged with a Zeiss light microscope equipped with an AxioCam MR3 camera (Carl Zeiss, Jena, Germany) using bright-field and the ZEN software (provided by the device manufacturer).

#### 2.4.6 Sedimentation tests

Sedimentation was visualized and quantified as described previously [21]. In short, yeast cells were harvested from fully grown shake flask cultures on SM with their corresponding carbon source, washed, and resuspended in SM to a biomass concentration of  $2\text{ g dry weight} \cdot \text{L}^{-1}$ . After homogenizing the suspension by vortexing, they were placed in test tubes and imaged after 5 and 10 min.

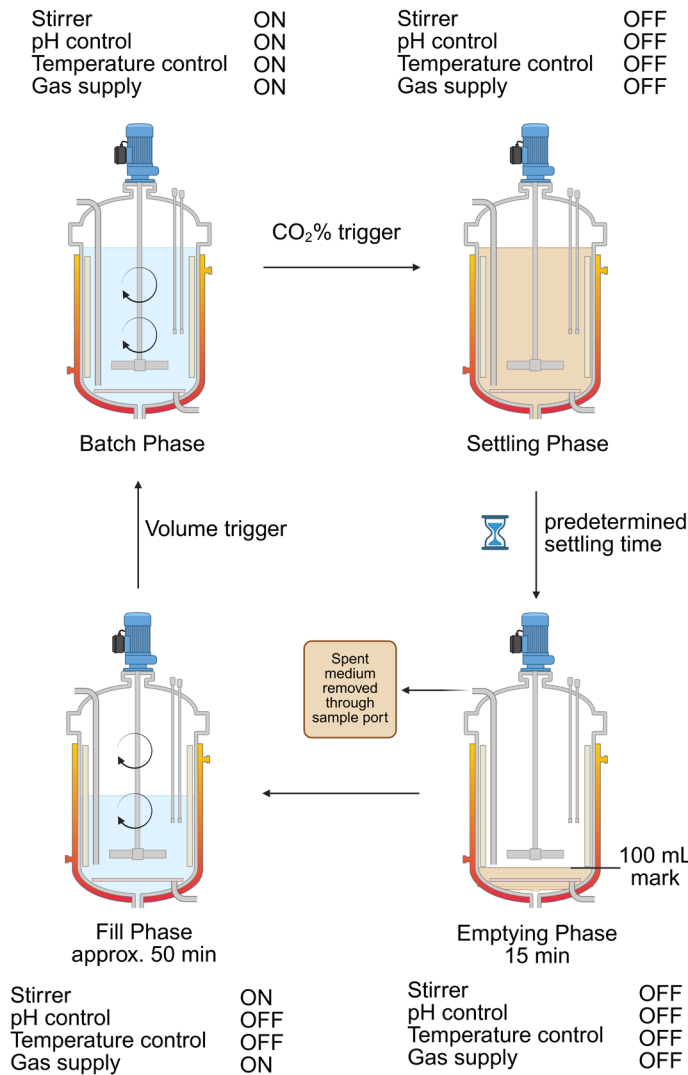
To quantify the sedimentation rate, biomass from fully grown shake-flask cultures was washed and resuspended in SM to a concentration of  $0.42\text{ g dry weight} \cdot \text{L}^{-1}$ . The suspension was incubated in a 1.5 mL cuvette for 30 min while the  $\text{OD}_{660}$  was continuously recorded using a Jenway 7200 Spectrophotometer (Bibby Scientific, Staffordshire, UK).

To simulate biomass retention inside a sequential batch reactor at a smaller scale, biomass from stationary-phase shake-flask cultures grown on SM with their corresponding carbon source was washed and resuspended in SM to a concentration of  $2 \text{ g dry weight} \cdot \text{L}^{-1}$ . Ten mL of this suspension was added to 15 mL tubes and briefly vortexed to ensure homogeneity. Either immediately or after 30 min settling time, 1-mL samples were carefully drawn at 1 min intervals from a fixed sampling height at the 1 mL mark of the tube and added to a second tube. The concentration of biomass in the remaining 1 mL was calculated by measuring biomass in the second tube and subtracting it from the total biomass.

#### 2.4.7 Batch and sequential batch cultivation

Anaerobic batch cultures were grown in 2-L laboratory bioreactors (Applikon, Delft, The Netherlands) with a working volume of 1 L, continuously sparged with  $0.5 \text{ L N}_2 \cdot \text{min}^{-1}$  and stirred at 400 rpm. Temperature was set to  $30^\circ\text{C}$  and pH was controlled at 5.0 by automated addition of 2 M KOH. Foaming was reduced by adding  $0.2 \text{ g} \cdot \text{L}^{-1}$  antifoam C (Sigma-Aldrich) to the medium. Precultures were centrifuged and resuspended in sterile demineralized water before inoculation  $0.2 \text{ g dry weight} \cdot \text{L}^{-1}$ . In the co-culture cultivations, the inoculum was a 1:1 mix of both strains, combined prior to inoculation. Bioreactor exhaust gas was cooled by a condenser to  $2^\circ\text{C}$ , dried with a Perma Pure dryer (Inacom Instruments, Veenendaal, the Netherlands) and  $\text{CO}_2$  concentrations were continuously recorded using an NGA 2000 analyser (Rosemount Analytical, Orrville, OH).

Sequential batch cultivation was set up by programming an automated empty-refill sequence, using the off-gas  $\text{CO}_2$  concentration as trigger (Fig. 2.6). In each cultivation cycle (Batch phase), stirrer and gas supply were turned off when the off-gas  $\text{CO}_2$  had reached 2 % of the maximum registered  $\text{CO}_2$  concentration during the cycle, indicating that the culture had exhausted the medium and entered stationary phase. The culture was then left to settle for a predetermined time (Settling phase), at which the stirrer and gas supply was turned off, as well as the pH and temperature control. Subsequently, 900 mL of spent medium was automatically removed via the sampling port (Emptying phase, approx. 15 min). The bioreactor was refilled using 900 mL fresh medium with the stirrer and gas supply re-activated (Fill phase, approx. 50 min) until a volume-based trigger was activated. After refilling, the temperature and pH control were re-activated, initiating a new batch phase. The cycle time was defined as the time from the start of the fill phase until reaching the settling phase. The reactor was operated in sequential batch mode for  $7 \pm 1$  cycles.



**Figure 2.6: Setup for sequential batch reactors with biomass settling phase.** The batch phase, in which the main fermentation occurred, was initiated at a liquid volume of 1 L with gas supply and stirrer, as well as pH and temperature control, active. After a drop in  $\text{CO}_2\%$  in the off-gas indicated full depletion of the carbon source(s), the settling phase was initiated automatically, and stirrer and gas supply were turned off. At the same time, pH and temperature control were turned off to prevent overheating or incorrect pH adjustment. After a pre-determined settling time (e.g. 30 min), 900 mL spent medium was removed through the sampling port (Emptying phase). Subsequently, 900 mL of fresh medium was added, which required ca. 50 min and was terminated automatically by a volume sensor (Fill phase). Here, stirrer and gas supply were re-activated. After reaching the working volume of 1 L, temperature and pH control were reactivated to initiate a new cycle. Figure was created in BioRender. Fecker, T. (2026): <https://BioRender.com/ds97o4y>

### 2.4.8 Analytical methods

Biomass dry weight was measured gravimetrically by filtering 10 mL samples over pre-weighed nitrocellulose filters (pore size 0.45  $\mu\text{m}$ ) in duplicates. Filters were washed with demineralized water and dried in a microwave oven for 20 min at 360 W. Specific growth rates were based on at least five biomass dry weight samples taken during the exponential growth phase.

Metabolite concentrations were determined by high-performance liquid chromatography on an Agilent 1260 Infinity HPLC system (Agilent Technologies, Santa Clara, CA), equipped with a Bio-Rad Aminex HPX 87H ion-exchange column (Bio-Rad, Hercules, CA) eluted at 60 °C with 2 mM  $\text{H}_2\text{SO}_4$  as mobile phase at a flow rate of 0.6 mL  $\cdot$  min<sup>-1</sup>. Metabolite concentrations were quantified using an Agilent G1362A refractive-index detector and an Agilent G1314F VWD detector.

### Acknowledgements

This work was funded by Delft University of Technology. The postdoc project of M.R. is funded by the Stevin Grant from the Netherlands Science Foundation (NWO) to J.T.P. We thank Floor Bleekveld, Sanne Diepen, and Fons Sluijter for their contributions to the project.

### Author contributions

TF: Validation, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. MR: Investigation, Writing – Review & Editing. JTP: Conceptualization, Supervision, Writing – Review & Editing. RJvT: Conceptualization, Supervision, Writing – Review & Editing.

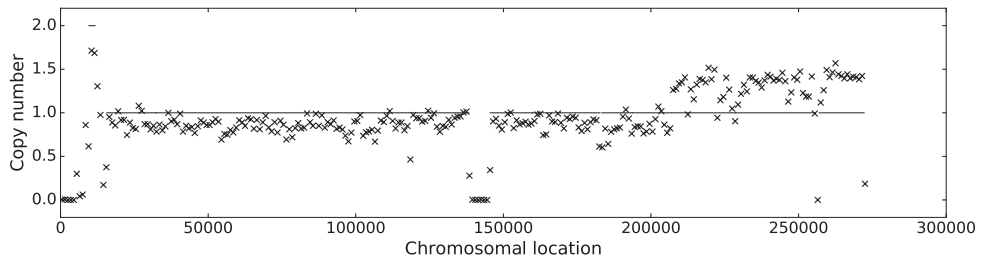
## Supplementary information

Supplementary table S2.1: Plasmids used in this study

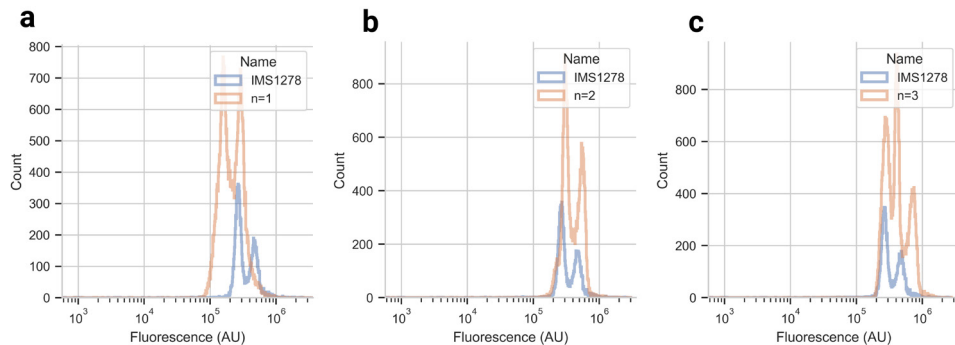
| Plasmid | Characteristics                                      | Reference |
|---------|------------------------------------------------------|-----------|
| pROS13  | 2 $\mu$ m ori, ampR, kanMX, gRNA-CAN1.Y, gRNA-ADE2.Y | [50]      |
| pUDR913 | 2 $\mu$ m ori, ampR, kanMX, gRNA-ACE2                | This work |

Supplementary table S2.2: Oligonucleotides used in this study. All oligonucleotides were purified by desalting.

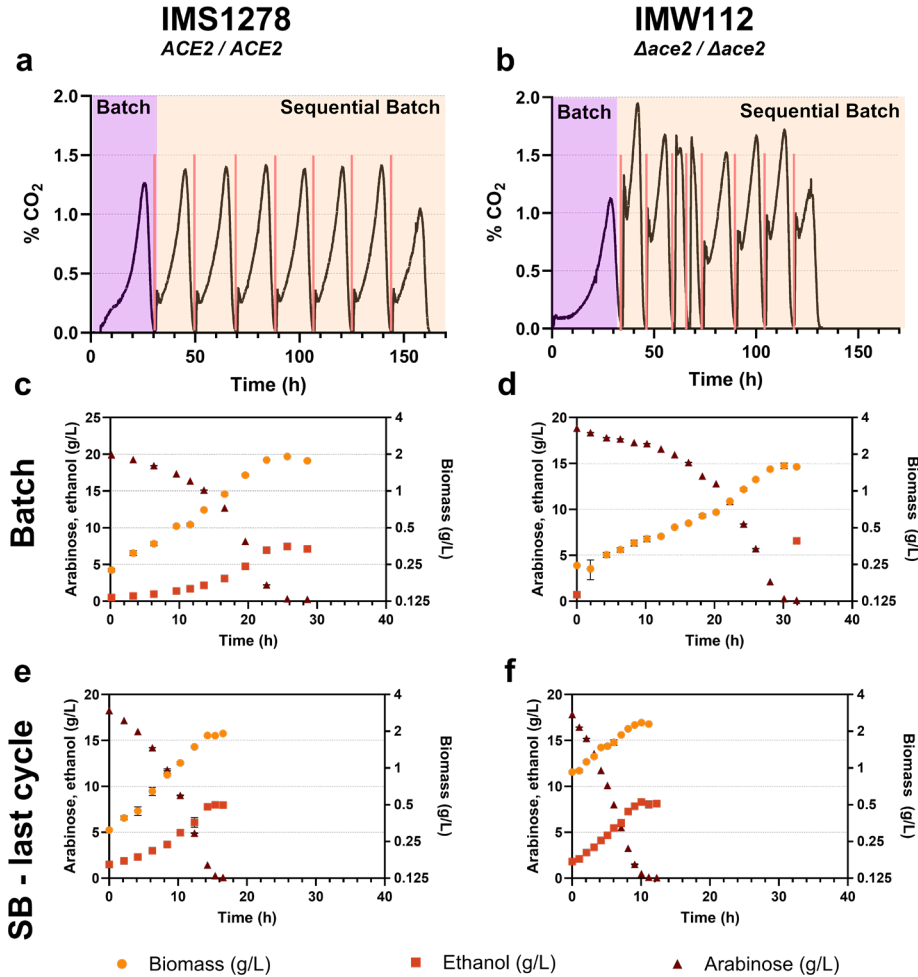
| Oligonucleotide name | Sequence                                                                                                                         |
|----------------------|----------------------------------------------------------------------------------------------------------------------------------|
| 6005                 | GATCAITTTATCTTTCACTGCGGAGAAG                                                                                                     |
| 20864                | TGCGCATGTTTCGGCGTTCGAACTTCTCCGCAGTGAAAGATAAATGATCATCC<br>GAGGCGATATTAACGTGTTTTAGAGCTAGAAATAGCAAGTTAAAATAAG                       |
| 20865                | ATCATGCATAGATAAATGTTCTGTCAGAGAGCATCAGTTTCGTTTGAAAGGGTGC<br>GGTTCGGTTATCCATGATTGTATTAACACCGTTTTTGGTCTATAGATTTCITT<br>AGTTATTCTTG  |
| 20866                | CAAGAAATAACTAAAGAAATCTATAGGACCAAAAACGGTGTTAATACAATCATG<br>GATAACCGAACCGCACCCCTTCAAACGAAACTGATGCTCTCTGACGAACATTTA<br>TCTATGCATGAT |
| 20867                | GATGCACCGTTGTATTTTCG                                                                                                             |
| 20868                | CCGTCCTCCATTAGAATCC                                                                                                              |



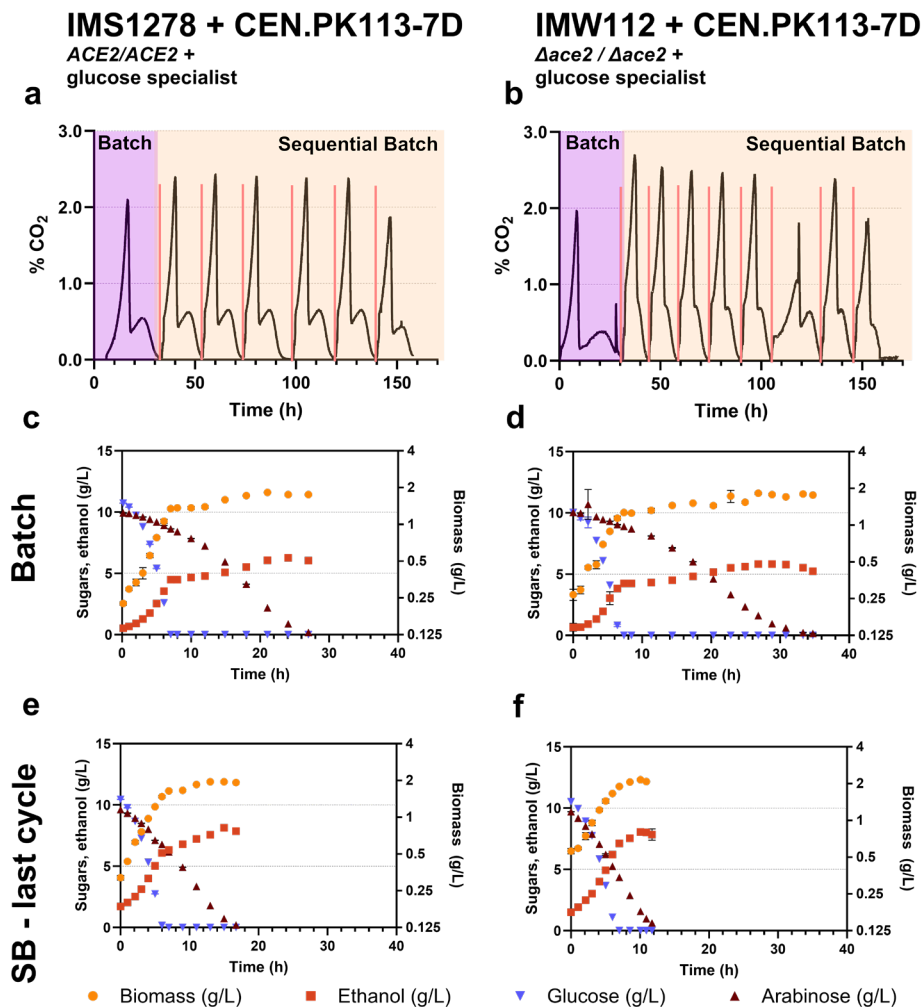
**Supplementary Figure S2.1: Computational analysis of sequencing coverage using the MAGNOLYA algorithm.** As a representative for all chromosomes chromosome VI is shown here. Within the algorithm, the coverage is mapped to a reference representing the average coverage of the whole genome. Partial duplications are indicated with a whole integer copy number (e.g. 2). In the above graph, there is a partial copy number change to ca. 1.5. Since the copy number must be an integer, this suggests that the whole genome had undergone duplication and the region from ca. 200,000 onwards has a copy number of  $n=3$ .



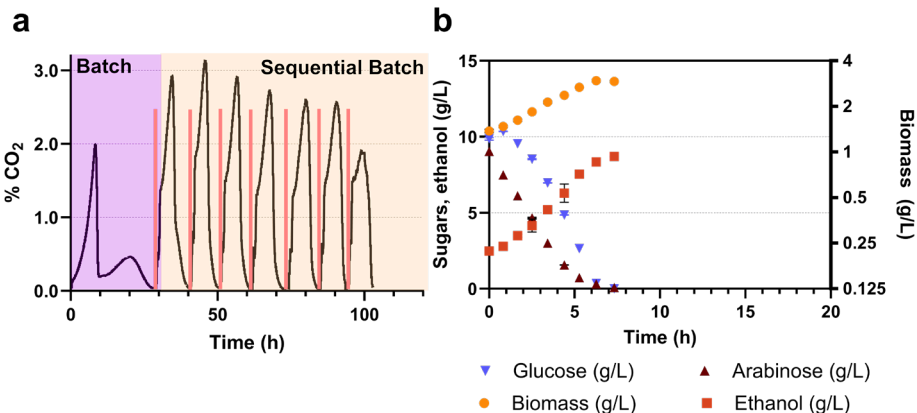
**Supplementary Figure S2.2: Ploidy test of the isolated strain IMS1278 against reference *S. cerevisiae* strains with haploid (CEN.PK 113-7D, left panel), diploid (CEN.PK122, middle panel) or triploid (FRY153, right panel) genomes.** The strains were stained with Sytox Green and then analysed using flow cytometry. The matching flow cytometry pattern showed that IMS1278 had a diploid genome.



**Supplementary Figure S2.3: Cluster formation increases biomass retention and arabinose consumption across cycles in a sequential batch setup.** Fermentation kinetics of diploid *S. cerevisiae* strains IMS1278 (Panels a,c,e; *ACE2/ACE2* arabinose-specialist strain, see Table 2.2 for genotype description) and IMW112 (Panels b,d,f; *Δace2/Δace2* mutant of strain IMS1278), grown in anaerobic sequential-batch bioreactors on SM with 20 g · L<sup>-1</sup> arabinose. a+b) CO<sub>2</sub> content of bioreactor off-gas during the initial batch culture and seven subsequent sequential batch cycles. Off-gas data for the final cycle are influenced by sampling for metabolite analyses. The 30-min settling phase is indicated in red. c+d) Concentrations of arabinose, biomass and ethanol in the initial anaerobic batch cultures of each strain. Cultures were inoculated with 0.2 g · L<sup>-1</sup> biomass. Due to an issue with the ethanol sampling procedure, only start and endpoint ethanol concentrations could be determined for the IMW112 strain. e+f) Concentrations of arabinose, biomass and ethanol in the seventh cycle of sequential-batch operation of each strain. Metabolite and biomass data shown in the figure represent average ± SD of technical replicates. Duplicate bioreactors were run for each strain, with data from the second one shown in the figure.



**Supplementary Figure S2.4: Selective sedimentation of a cluster-forming arabinose-specialist strain accelerates conversion of a glucose-arabinose mixture in sequential batch co-cultures.** Fermentation kinetics of co-cultures of the pentose-non-fermenting reference strain ('glucose specialist') *S. cerevisiae* CEN.PK113-7D with either strain IMS1278 (panels a,c,e; *ACE2/ACE2* arabinose - specialist strain, see Table 2 for genotype description) or IMW112 (panels b,d,f;  $\Delta ace2/\Delta ace2$  mutant of strain IMS1278), grown in anaerobic sequential-batch bioreactors on SM with  $10 \text{ g} \cdot \text{L}^{-1}$  each of D-glucose and L-arabinose. a+b)  $\text{CO}_2$  content of bioreactor off-gas during and initial batch culture and subsequent sequential-batch cycles. Off-gas data for the final cycles are influenced by sampling for metabolite analyses. The 30-min settling phase is indicated in red. c+d) Concentrations of arabinose, biomass and ethanol in the initial anaerobic batch cultures of each co-culture, inoculated with  $0.1 \text{ g} \cdot \text{L}^{-1}$  of each strain. e+f) Concentrations of arabinose, biomass and ethanol in the final cycle of sequential-batch operation. Metabolite and biomass data shown in the figure represent average  $\pm$  SD of technical replicates. Duplicate bioreactors were run for each strain, with data from the second one shown in the figure.



**Supplementary Figure S2.5: Extension of settling time results in simultaneous conversion of glucose and arabinose in a sequential batch co-culture.** Fermentation kinetics of co-cultures of the pentose-non-fermenting reference strain ('glucose specialist') *S. cerevisiae* CEN.PK113-7D and the arabinose - specialist strain IMW112 (*ace2/Δace2*) grown in anaerobic sequential-batch bioreactors on SM with 10 g · L<sup>-1</sup> each of D-glucose and L-arabinose. In comparison with the experiments shown in Figure 4, settling time after substrate depletion was extended from 30 to 60 min. a) CO<sub>2</sub> content of bioreactor off-gas during and initial batch culture and subsequent sequential-batch cycles. Off-gas data for the final cycle are influenced by sampling for metabolite analyses. The 60-min settling phase is indicated in red. b) Concentrations of arabinose, glucose, biomass, and ethanol in the final cycle of sequential-batch operation. Metabolite and biomass data shown in the figure represent average ± SD of technical replicates. Duplicate bioreactors were run for each strain, with data from the second one shown in the figure.

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*Alles ist Wechselwirkung.*

*Everything is interaction.*

*Alexander von Humboldt (1803)*

*Diary entry during his South America expedition*



# Chapter 3

## **Time of first contact determines cooperator success in a three-member microbial consortium**

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Essentially as published in ISME Communications (2025), 5(1):1-10  
<https://doi.org/10.1093/ismeco/ycaf004>

## Abstract

Microbial communities are characterized by complex interaction, including cooperation and cheating, which have significant ecological and applied implications. However, the factors determining the success of cooperators in the presence of cheaters remain poorly understood. Here, we investigate the dynamics of cooperative interactions in a consortium consisting of a cross-feeding pair and a cheater strain using individual-based simulations and an engineered *L. cremoris* toy consortium. Our simulations reveal first contact time between cooperators as a critical predictor for cooperator success. By manipulating the relative distances between cooperators and cheaters or the background growth rates, influenced by the cost of cooperation, we can modulate this first contact time and influence cooperator success. Our study underscores the importance of cooperators coming into contact with each other on time, which provides a simple and generalizable framework for understanding and designing cooperative interactions in microbial communities. These findings contribute to our understanding of cross-feeding dynamics and offer practical insights for synthetic and biotechnological applications.

### 3.1 Introduction

Microorganisms often live together in surface-attached communities of many strains and species, called biofilms. An early stage of the planktonic cell to biofilm life cycle is microcolonies, which make up the initial kernels that later grow into larger biofilms [1]. Studying the formation of these microcolonies provides valuable insight into biofilm development [2]. The organisms living in biofilms can form complicated networks of antagonistic, mutualistic, competing or cooperating interactions [3–5]. One of the ways species can cooperate is by cross-feeding, where both species produce metabolites or other essential compounds that benefit the other [6]. The production of these compounds often comes at a cost for the producer in the form of extra expended energy (e.g. in the form of ATP). This creates opportunities for cheater species to exploit cooperators, by reaping the same benefits from the cross-fed compounds without contributing to the cooperation themselves [7]. Studying the interactions in bacterial systems can have implications for applications concerning biofilms in medical, biotechnological, and industrial contexts [8–10]. Moreover, because mutualism and cooperative interactions can be observed in all branches of the phylogenetic tree, there is general interest in what makes this type of social behavior evolutionary stable [7]. Due to their relative simplicity, microbial systems can be used as model systems for studying broader social behaviors in biology [11]. The interactions between microorganisms are in general complex, with many inter-dependent variables that are difficult to isolate [12]. An important factor on the dynamics of any collection of interacting species is spatial organization [13–18]. This spatial structure plays a role in both the emergence and the maintenance of cooperator co-existence [19]. For example, spatially structured environments can promote clonal patching, which is useful for intra-species cooperation [20,21] as well as pattern formation in multispecies colonies [16,22].

In this paper, we employ individual-based modelling (IBM) to simulate a consortium of two cooperating species and a selfish cheater growing on a surface. By incorporating spatial structure and proximity in metabolic interactions, we attempt to uncover the factors that govern cooperative success in the face of cheating [23]. This will contribute to our understanding of population dynamics in cooperative biological systems.

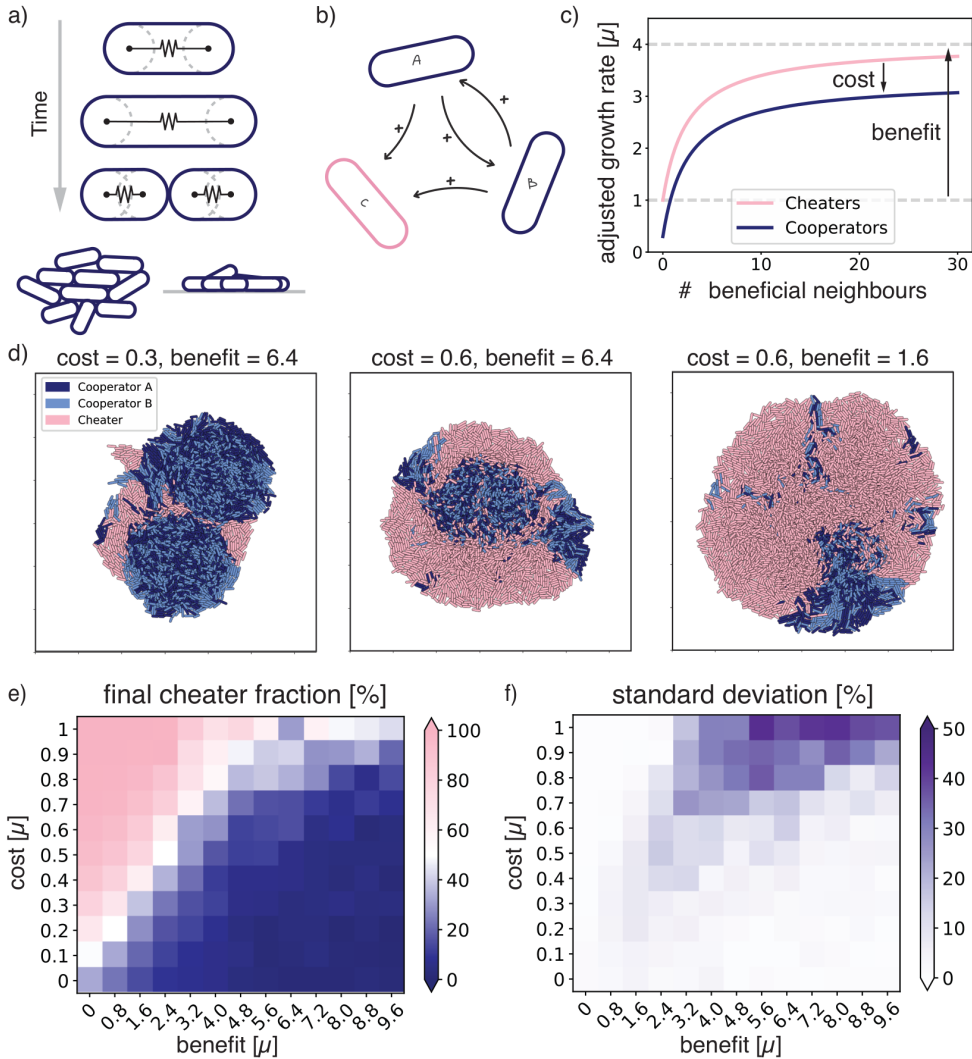
## 3.2 Results and discussion

### 3.2.1 Cooperator success is highly dependent on initial placement of particles

To explore the impact of local cross-feeding interactions on the development of a multi-species microcolony, we modelled interacting particles growing on a surface (Figure 3.1a). We defined three different species: two cooperators A and B, and a cheater C (Figure 3.1b). We made use of the fact that metabolic interactions have been shown to take place at very short distances and have the particles adjust their growth rate based on the number of their beneficial nearest neighbors [24,25]. The strength of the cross-feeding interaction between cooperators A and B is characterized by two main factors: the metabolic cost the cooperators pay to contribute to the cooperation, and the added growth benefit they experience from this interaction. The cheaters do not pay the cost but experience the same benefit (Figure 3.1c). For now, the interaction with the cooperators is symmetric, in the sense that the cheater responds similarly to cooperator A as to cooperator B and it needs both to grow. Later in this paper, we also explore asymmetric cheater interactions. The particles are spherocylindrical particles that grow and divide. We start each run with 10 of each species randomly distributed on a surface. We then let them grow until the colonies reach a size of  $10^4$  particles.

In order to understand how the strength of the local interactions shaped the development of the microcolony, we simulated growing colonies for varying costs and benefits and recorded the cheater fraction. Typical simulation snapshots of the final colonies are shown in Figure 3.1c. The final cheater fractions, averaged over 15 runs, are shown in Figure 3.1e. The bottom-left data point represents a situation with no cost and no benefit, which means that the particles are not interacting at all and their growth rate remains the same. As the cost of cooperation increases while the benefit stays zero, the final cheater fraction increases. In turn, increasing the benefit decreases the cheater fraction and favors the cooperators. Interestingly, there are not many intermediate values for the final cheater fraction, showing more switch-like behavior than would be expected from a well-mixed system (Supplementary Figure S3.2).

Surprisingly, the simulations gave a large variation between runs with the same interaction parameters, particularly for higher costs and benefits (Figure 3.1f). Here, we found standard deviations of up to 50%, which indicates that the final cheater fraction can be anywhere between 0% and 100% for simulations with the same interaction input parameters. Because the only difference between these runs was the initialization of the 30 particles at the start of the run, the large variance had to be a result of the initial placement of the particles, which we explore next.



**Figure 3.1: Effect of cost and benefit of cooperation on cooperator success in individual-based simulations.** a) Schematic representation of the individual-based model. Spherocylindrical particles grow and divide, forming a 3D colony. b) The simulated consortium of two cooperators, A and B, that benefit each other and a cheater C, which benefits from both cooperators A and B but does not reciprocate. c) Growth rate of both the cooperators and the cheaters, dependent on the number of beneficial neighbors (i.e. neighbors that provide a useful compound). The growth rate is affected by the cost and benefit of cooperation. d) Snapshots of the final 3D colonies for different combinations of cost and benefit. Area shown is always  $130 \times 130 \mu\text{m}^2$ . e) Final cheater fractions for varying cost and benefit, with averages taken over 15 runs. f) The standard deviation of those runs.

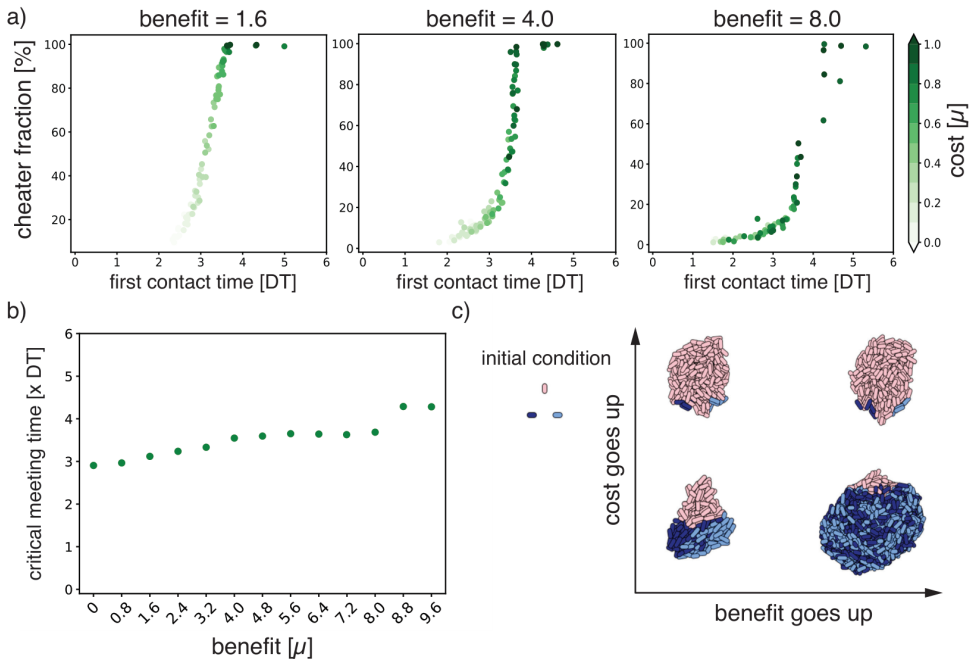
## 2.2 First contact time determines cooperator success

In order to investigate how the initial placement of the particles leads to different final cheater fractions, we visually inspected the resulting colonies of the simulations. In general, we observed that colonies were fairly segregated, akin to clonal patches observed in 2D colonies [9]. The cooperator patches are often well mixed, with equal amounts of A and B, which we expect is due to a combination of nematic mixing and the mutualism between these species [20,26,27]. In different simulations with the same input parameters, we observed a varying number of these patches of mixed cooperators (Supplementary Figure S3.1). Because the cooperators depend on each other's proximity to cross-feed, we speculated that the final cheater fractions might be determined by how likely it is for cooperators to meet.

In order to investigate this relation between the likelihood of meeting and final cheater fraction, we took all the runs and we recorded the first time point where a cooperator A particle comes into contact with a cooperator B particle. Plotting the final cheater fraction as a function of these first contact times for each value of the benefit yielded a clearly defined curve with comparatively little variance (Figure 3.2a). Therefore, for a given benefit, the time of first contact was a more informative predictor for cooperator success than the cost of cooperation. This dependence on cooperators encountering each other is in line with earlier findings on the importance of co-localization probabilities in a similar consortium growing in microdroplets [28]. Other work on two-species cooperator-defector consortia growing plates showed the importance of founder cell configuration [29]. Additionally, expansion-collision dynamics and initial distance between microorganisms have been shown to be important factors for cooperator-defector co-existence [30]. Although all these systems are not identical, they do point to a general principle in cooperative dynamics. To explore this further, for each value of the benefit, we determined a critical meeting time before which A and B need to make contact in order to outcompete the cheaters and make up >50% of the final population (Figure 3.2b). If the first contact time occurred after this point, cheaters dominated the final colony of  $10^4$  particles. The critical meeting time depended on the cooperator benefit, where a higher benefit allowed for a later critical meeting time. In this case, once first contact had been made, cooperators were able to make up for lost time by growing faster once they were together. Conversely, if the cooperator benefit was lower, the cooperators needed to meet earlier to outcompete the cheaters.

It is important to note that first contact time is determined not only by the cost and benefit, but also the relative distance between the cooperators in the initial placement. To illustrate this, we visualized the outcome of simulations with a well-defined initial placement of the three particles (Figure 3.2c). Here, the initial distance between the particles is always the same, and out of range from each other. The

cost then affects the time it takes for the particles to traverse that distance, as the cost affects the growth rate of the particles when they are on their own (i.e. their background growth). A higher cost causes the cooperators A and B to have a lower background growth rate, so they are slower to reach each other. If they are too slow, as shown in the top-left, cheater particles grow in between them before they get the chance to make contact. In this case, having a higher benefit (top-right) will not help the cooperators as they never get to reap the rewards of their cooperation. If the cost is lower and A and B find each other before the critical meeting time (bottom-left), they can compete effectively with the cheaters. When the benefit becomes larger (bottom-right), they can significantly outcompete the cheaters once they meet. In our system of random initial distances between particles, this interplay between distance and background growth is what leads to the high variation in competitive outcome. From these simulation results, we propose that it is not just the initial distances between cooperators or just the details of the cooperative interaction, but it is the first contact time that determines cooperator success in surface-attached colonies.

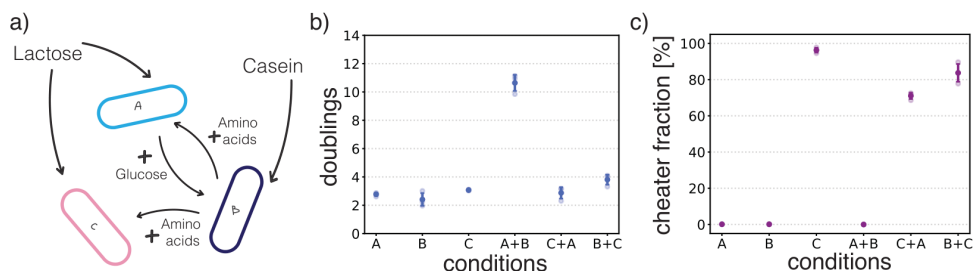


**Figure 3.2: First contact time determines cooperator success.** a) Final cheater fraction of single runs plotted as a function of first encounter time (measured in  $DT = 10^5$  simulation time steps) for three different benefits. Cost is denoted in green. b) Critical meeting time as a function of benefit. c) Toy system for a well-defined initial condition of three particles positioned in a triangle and the resulting colonies, for the combinations of two values for the cost (0.2 and 0.7) and two values for the benefit (5.6 and 8.8).

### 3.2.3 Experimental consortium of engineered *Lactococcus cremoris*

To test if the first contact time between cooperator species is also critical in a biological system, we attempted to translate our simulated consortium to a laboratory setting. We constructed a consortium of three *L. cremoris* strains (Figure 3.3a, Table 3.1) growing on agar plates containing lactose as a carbon source and casein as an amino acid source. Cooperator A, NZ9000 Lac<sup>+</sup> Glc<sup>-</sup>, can metabolize the galactose moiety of lactose, but exports the glucose moiety from the cell. The glucose can then be used by cooperator B, MG610, which cannot metabolize lactose. In return, MG610 expresses an extracellular enzyme to break down the casein into amino acids; this benefits A, which cannot metabolize the casein. Lastly, we engineered a cheater C, MG1363\_GFP Lac<sup>+</sup>, by transforming a plasmid containing the genes necessary to metabolize lactose into MG1363\_GFP. This constructed cheater can metabolize lactose and both resulting moieties, but, similar to A, lacks the enzyme to degrade casein. C also expresses GFP, which we use to determine the cheater fraction.

We grew A, B, and C individually on agar plates, and measured the total growth by comparing the final cell count to the initial cell count and calculating the number of times the cells had doubled. On the single strain plates, there was very little growth, which is what we expected from the designed cross-feeding interaction (Figure 3.3b). When growing all the pairwise combinations, we observed that A+B massively outgrew the other combinations. From this, we concluded that, as expected, these two strains experience a strong mutual benefit from growing together. Similarly, we concluded that on plates with C+A and B+C, there is no such mutualism, as the total cell abundance, measured in number of doublings, stalled. Furthermore, when we measured cheater fractions for these combined plates, we saw that C outgrows both A and B in a pairwise combination, pointing to a general advantage in growth rate that C has over both A and B (Figure 3.3c). All in all, we concluded that our experimental consortium accurately represents a cross-feeding pair together with a cheater.

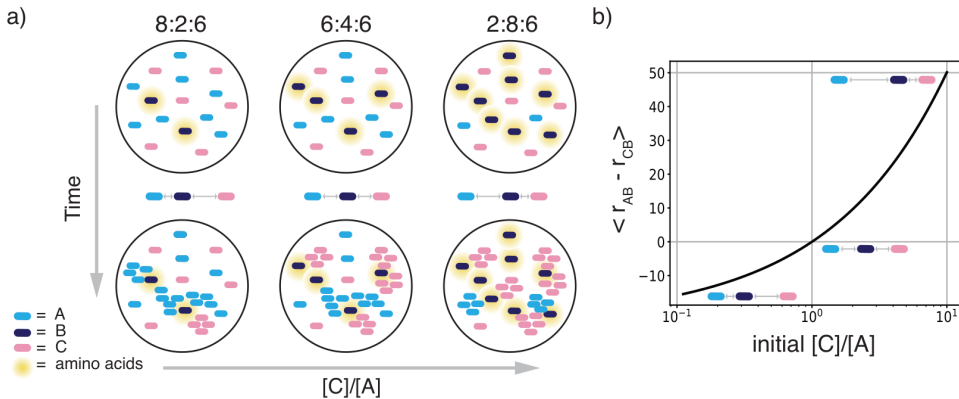


**Figure 3.3: Engineered consortium on agar plates.** a) *L. cremoris* consortium, consisting of cooperators A and B and cheater C. b) Control experiments showing the amount of doublings for single strain plates and pairwise combinations after three days incubation. c) Control experiments showing cheater fractions for all single strain plates and pairwise combinations. All conditions are measured in triplicate.

### 3.2.4 Relative average distance between cooperators determines cooperator success in plate experiments

To investigate how the first contact time would affect our experimental system, we needed a way to modulate the distance between cooperators on the plates. Unfortunately, it is not so simple to closely control the initial placement or the respective growth rates of the microorganisms on plates. Therefore, we needed a different way to adjust the chance of cooperators meeting each other. Unlike our simulated cheater, the cheater in our experimental consortium is not symmetric in the sense that it does not benefit as much from cooperator A as it does from cooperator B. Because both cooperator A and the cheater C need cooperator B for their amino acid production, we argue that the relative difference between the distance between B and A ( $r_{AB}$ ) and the distance between B and C ( $r_{CB}$ ) sets the chance of cooperators meeting. In essence, it is a race between A and C to reach B first.

To modulate the relative distances between A and B, and between C and B, we adjusted the ratio of  $[C]/[A]$ . When  $[C]/[A]$  is small ( $< 1$ ), there is more A on the plate than C. In this case, the chance of B finding itself close to an A is larger than B finding itself close to a C. If  $[C]/[A]$  is large ( $> 1$ ), the reverse is true, so on average it is more likely for a B to be surrounded by cheaters (Figure 3.4a). The relationship between  $[C]/[A]$  and the expectation value of the relative distance can be derived analytically (see Section S3.1 of the supplementary material and Figure 3.4b).



**Figure 3.4:  $[C]/[A]$  ratio as a proxy for relative cooperator–cheater distances.** a) Schematic diagram showing how changing the A:B:C ratios is expected to affect the first contact time between cooperators A and B. b) Relation between the expected average difference between the distance from B to A ( $r_{AB}$ ) and from B to C ( $r_{CB}$ ) to the  $[C]/[A]$  ratio. For the derivation, see Section S1 of the supplementary material.

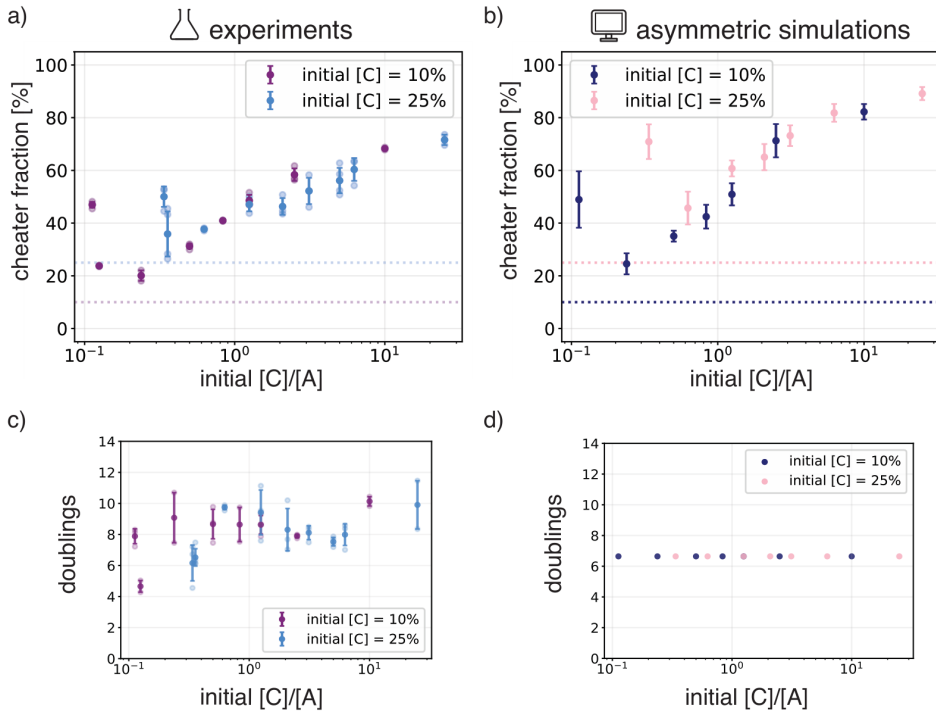
From our simulation results, we expected the final cheater fraction to rise with a higher relative distance between cooperators, as it would take longer for them to find each other. To test if this would also occur in our experimental consortium, we inoculated cells on plates in different A:B:C ratios, and after three days of incubation we washed the plates and measured the total cell count and the final cheater fraction (Figure 3.5a and b). Consistent with our expectations, a small  $[C]/[A]$  resulted in final cheater fractions of as low as 20%, where when the ratio was high, the final cheater fraction increased to almost 70%. Note that the total amount of cells after incubation was similar for all starting ratios (Figure 3.5c and d).

We show two sets of experiments, one where the initial cheater fraction is always 10% and one where it is 25%. Apart from the leftmost points of the respective curves (discussed below), they fall on to the same curve. From this, we concluded that, regardless of initial cheater fraction, the relative abundance of A and C and therefore the relative average distance between cooperators, determines cooperator success or failure in our experimental consortium.

### 3.2.5 Lowering the background growth of cooperators results in higher cheater fractions

The symmetric simulations demonstrated that not only the initial distance between cooperators determined cooperator success, but also how fast this distance could be traversed (Figure 3.2c). This speed is set by the background growth of the particles. In the simulation, we can set the background growth of the cooperator particles by changing the cost of cooperation (Figure 3.2c). A higher cost for cooperators means lower background growth, which we expected to result in a shift of the curve to the left, where more of cooperator A (i.e. a smaller  $[C]/[A]$  ratio) would be needed to achieve the same final cheater fraction. Indeed, increasing the cost in the asymmetric simulations slightly results in a shift to the left, shown as the blue data points in Figure 3.6b.

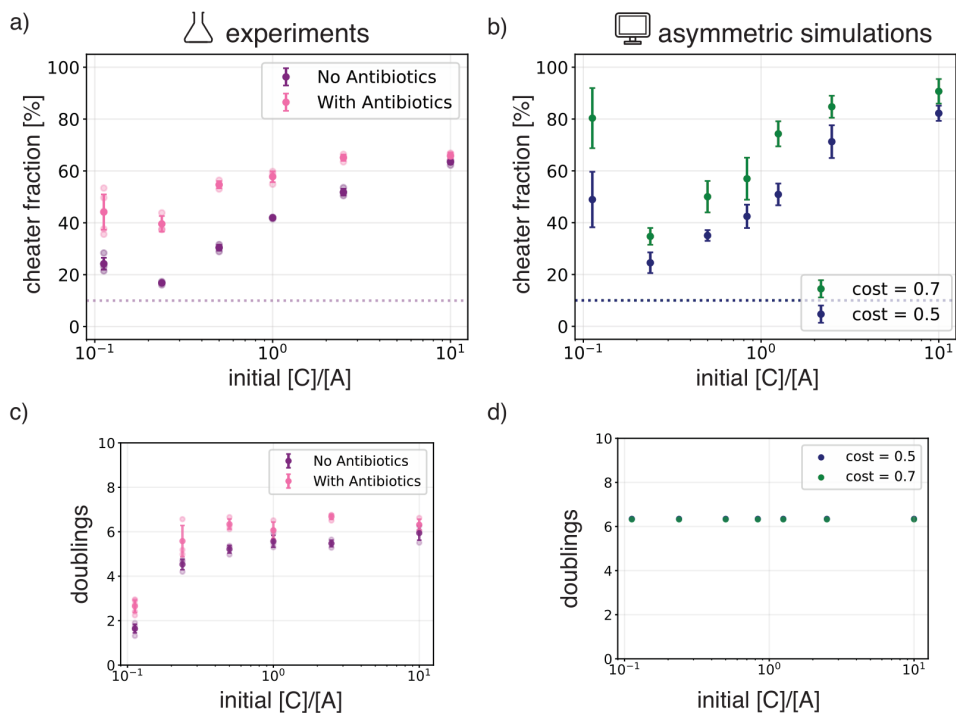
Again, we attempted to test these results from the simulations experimentally. While the cost and benefit of cooperation could not be directly altered, we hypothesized that by selectively inhibiting cooperator A we could achieve a similar effect. To that end, we used erythromycin, which is an antibiotic that only affects the growth of A as both B and C are resistant. We tested for a concentration of erythromycin that would only slightly inhibit the growth of A, which we found to be  $0.04 \mu\text{g ml}^{-1}$  (Supplementary Figure S3.4). We then performed the same experiment for the 10% initial cheater fraction on both normal (untreated) plates and on plates containing the antibiotic (Figure 3.6a). For the final cheater fraction on the antibiotic plates, we observed a shift to the left compared with the normal plates, where the same final cheater fraction on standard plates could be achieved on antibiotic plates with a lower ratio of  $[C]/[A]$ .



**Figure 3.5: Final cheater fractions depend on initial A:B:C ratios in experiments and simulations.** a) Final cheater fraction of plate experiments with different initial starting ratios of A:B:C, with a constant starting fraction of 10% C in purple and 25% C in blue. Error bars are standard deviation for three replicates. b) Final cheater fraction for simulations with the same starting ratios of A:B:C. Simulations are of an asymmetric interaction where A and B interact as before, but C only benefits from B. The cost and benefit for these runs are 0.5 and 2.4, respectively. Number of times the cells doubled between the time of initialization and measurement c) on the plates and d) in the simulations.

Note that in the simulations, we increased the cost for both A and B where, in the experiments, the antibiotic only affects cooperator A. Regardless, we achieved good agreement between the experimental and simulated data. This demonstrates that increasing the cost of cooperation or decreasing the background growth of the cooperators results in higher cheater fractions. Together with our previous results, we propose that this is because the background growth sets the first contact time for the cooperators, which is the main determinant for cooperator success.

The general concept of first contact times being instrumental in cooperator success suggests several strategies for cooperators to increase their survival rate. For instance, cooperators could increase their affinity for each other in solution, forming mixed aggregates. These would then function as the kernels that establish colonies elsewhere, which would greatly improve their chance of survival [1]. Interestingly, the spontaneous developing of cell-cell affinity has been previously shown in



**Figure 3.6: Higher cost of cooperation leads to higher cheater fractions.** a) Final cheater fraction for experiments with different [C]/[A] initial ratios on plates containing 0.04  $\mu\text{g ml}^{-1}$  erythromycin (in pink) and plates without erythromycin (in purple). b) Final cheater fraction for asymmetric simulations of different [C]/[A] initial ratios for two values of the cost. The benefit is 2.4 and all data points are averages and standard deviations of five runs. Number of times the cells doubled between the time of initialization and measurement c) on the plates and d) in the simulations.

mutualistic strains of *E. coli* [16]. Alternatively, chemotactic motility would be a way for cooperators that are already on a surface to establish contact early on, thereby ensuring a beneficial cooperative environment. Another strategy would be for cooperators to economize on the production of the cross-fed compound when growing on their own, and using quorum sensing to only start production when encountering other cells [31,32]. Both these strategies could also be explored in synthetic systems making use of specific adhesins and quorum sensing pathways [33,34].

Not only can the first contact time be exploited by cooperators, it can also provide tactics in a biotechnological application. An example of a widely applied cross-feeding interaction is the yoghurt consortium. Here, protease negative strains can form, which act as a cheater, being able to take over the protease positive strain. Our framework explains why this is less of a problem in a surface-attached or otherwise spatially structured environment [35]. In kombucha production, aggregation

between interacting species has a positive effect on the yield [36,37]. We can now hypothesize that this is due to the metabolically interacting species being in contact early on in the process, outcompeting other strains present and increasing overall yield. Although this should be further tested and verified, the framework proposed in this work should be applicable to similar consortia of different species.

Finally, what this work also shows is that approximating metabolic interactions by only considering the nearest neighbors is a valid way of modelling cross-feeding. Even though our model is quite simple, it is able to capture and predict the behavior of a real system that is much more complex and has many more interdependent variables than we include in the model. For example, the species in our simulations only differ from each other in how they respond to their neighbors, whereas the strains in our experiment also show variability in overall growth rate. Moreover, in our experiments the nutrient density probably changes over time as the plates grow more dense. Regardless, our simple model of metabolic interactions on a surface is capable of capturing important features of the real system, providing insight into cross-feeding in the presence of cheating.

### 3. Summary

Microbial collaboration is an abundant phenomenon with ecological and applied relevance, yet the factors contributing to cooperator success in the presence of cheaters are poorly understood. We set out to investigate the factors contributing to cooperator success in the presence of an exploiting cheater growing together on a surface. Individual-based simulations of a consortium consisting of three species—a cross-feeding pair and a cheater—indicated a strong influence of the initial placement of the microorganisms on the final outcome. Focusing on the mechanisms, we demonstrated that first contact time was a better predictor for cooperator success than the value of the interaction parameters alone. We then showed how a combination of cost and initial placement together affect this first contact time.

We translated our simulations to an engineered *L. cremoris* toy consortium, consisting of two mutualistic strains and a cheater strain growing on agar plates. We show that by changing the relative distance between cooperators and cheaters by altering the starting ratios of A, B, and C, we could directly influence cooperator success.

We recreated our experimental findings by a simple adjustment to the model, making the cheater an asymmetric cheater, further showing how the average distance between cooperators is responsible for cooperator success.

Finally, we showed that, next to the relative distance between cooperators, the time it takes to traverse that distance affects the final cheater fraction as well. This time is set by the background growth, which is dependent on the cost of cooperation in

the simulations. In the experiments, we used antibiotics to selectively inhibit the background growth of cooperator A, giving good agreement with the simulations.

We conclude that in a cross-feeding cooperative interaction between strain A and strain B, the ability to find each other on time is the determining factor in cooperator success in the presence of a cheater strain.

Additionally, we have shown that metabolic cross-feeding can be modelled by adjusting the growth rate of particles depending on their nearest-neighbors. Our findings provide better understanding of cross-feeding dynamics in surface-attached communities, as well as an intuitive framework for designing and altering cross-feeding consortia in synthetic and biotechnological applications.

### 3.4. Materials and methods

#### 3.4.1 Individual-based model

In our simulations, we initialize spherocylindrical particles on a surface and let them grow, divide, and interact with each other [38]. The mechanical interactions of the particles are the same for all species: in addition to steric repulsion between the particles, they have an attractive potential, which makes them stick together and to the surface (see Section S3.1 of the supplementary material). The cross-feeding interaction between the different species is implemented by adjusting their growth rates based on their immediate environment (Figure 3.1c) [24,25]. In particular, for every growth step, we count for each particle how many beneficial neighbors they have in their immediate vicinity and use this number as input for a scaled Monod equation:

$$\mu'_A = \mu'_B = \mu \cdot \left( 1 + \frac{n}{K_s + n} \cdot b - c \right),$$

$$\mu'_C = \mu \left( 1 + \frac{n}{K_s + n} b \right)$$

which determines the growth rate of the particle,  $\mu'$ . Here,  $n$  is the number of beneficial neighbors,  $K_s$  is the Hill coefficient, which we set to 2.5 as this is the average number of beneficial direct neighbours in a well-mixed colony of  $10^4$  particles, and  $\mu$  is the background growth of the cheaters (i.e. the growth rate of an isolated cheater particle). Both benefit,  $b$ , and cost,  $c$ , are given as fractions of  $\mu$ , where  $b$  sets the maximal growth rate a particle can achieve and  $c$  gives a downward shift, which can have any value between 0 and 1.

### 3.4.2 Cross-feeding simulations

We implement a symmetric cross-feeding interaction by having species A and B increase their growth rate depending on how many of their neighbors are B and A, respectively. C increases its growth rate based on the minimum number of A and B in its vicinity. At the beginning of the simulation, 10 particles of each species are randomly initialized on a  $50 \times 50 \mu\text{m}^2$  patch of surface. We grow the system to a final size of  $10^4$  particles.

### 3.4.3 Asymmetric simulations

For the asymmetric case that resembles our experimental consortium, the interactions remain the same except that, for the growth rate of C,  $n$  is taken to be the number of B particles in the neighborhood. Additionally, 100 particles are initialized in different A:B:C ratios, and the system is grown to a final size of  $7 \times 10^3$  particles.

### 3.4.4 Microbial strains and growth conditions

Strains and plasmids used in this study are listed in Table 3.1. All strains were grown on CDMpc, described by Price et al. [39] at  $30^\circ\text{C}$  in a stationary incubator. We used glucose and lactose as carbon sources, in the concentrations as indicated. Where indicated, the strains were grown on CDMpccas, containing 0.2 wt% casein sodium salt (from bovine milk, #C8654, Sigma Aldrich, Saint Louis, MO, USA) instead of amino acids. To prepare agar plates, liquid medium was supplemented with 2 wt% BD Difco™ Bacto™ Agar (BD, NJ, USA).

*L. cremoris* NZ9000 Glc<sup>-</sup> Lac<sup>+</sup> [40], *L. cremoris* MG5267 and MG1363\_GFP Lac<sup>+</sup> (this study) were precultured in 25 ml CDMpc + 0.09 wt% lactose, and *L. cremoris* MG610 [41] and MG1363\_GFP [42] were precultured in 25 ml CDMpc + 0.09 wt% glucose. Freezer stocks were prepared by growing the strains in CDMpc with the appropriate carbon source and storing the stationary culture at  $-80^\circ\text{C}$  with 20 vol% glycerol. Where indicated, erythromycin (Sigma-Aldrich, 856193, Saint Louis, MO, USA) was added at the indicated concentration. For the isolation of pMG820 and transformation into MG1363\_GFP, M17 medium was used (from powder, Thermo Fisher Scientific, MA, USA).

**Table 3.1. Strains and plasmids used in this study**

|                                          | Description                                                                                        | Reference  |
|------------------------------------------|----------------------------------------------------------------------------------------------------|------------|
| <b>Strains</b>                           |                                                                                                    |            |
| NZ9000 Glc <sup>-</sup> Lac <sup>+</sup> | <i>L. cremoris</i> NZ9000 $\Delta glk \Delta ptnABCD$ , carrying pMG820                            | [40]       |
| MG610                                    | <i>L. cremoris</i> MG1363 with two prtMP copies integrated into the genome; erythromycin resistant | [41]       |
| MG1363_GFP                               | <i>L. cremoris</i> MG1363 with Dasher-GFP gene integrated into the genome; erythromycin resistant  | [42]       |
| MG1363_GFP Lac <sup>+</sup>              | MG1363_GFP carrying pMG820; erythromycin resistant                                                 | This study |
| MG5267                                   | <i>L. cremoris</i> MG1363 with lactose operon integrated into the genome                           | [43]       |
| <b>Plasmids</b>                          |                                                                                                    |            |
| pMG820                                   | 23.7 kb lactose miniplasmid containing <i>lacFEGABCD</i>                                           | [44]       |

### 3.4.5 Isolation of pMG820 and transformation into MG1363\_GFP

To construct MG1363\_GFP Lac<sup>+</sup>, pMG820 was isolated from 5 ml of a stationary NZ9000 Glc<sup>-</sup> Lac<sup>+</sup> culture, cultivated in M17 + 0.5 wt% lactose, as follows. The culture was centrifuged using a Sorvall ST40 centrifuge (Thermo Fisher Scientific, MA, USA) (10 min, 5000 g), and resuspended in 30 mM Tris-HCl pH 8, 3 mM MgCl<sub>2</sub>, 25 wt% sucrose and 2 mg · ml<sup>-1</sup> lysozyme (from egg white, 10837059001 Roche, Basel, Switzerland) and incubated for 30 min at 37 °C. Afterwards, the plasmid was isolated using the Gene- JET Plasmid Miniprep Kit (Thermo Fisher Scientific, MA, USA). Once obtained, the plasmid was transformed into MG1363\_GFP as described by Wells et al. [45] with the following adaptations. The MG1363\_GFP cells were precultured in 50 ml M17 broth with 17 wt% (0.5 M) sucrose, 2.5 wt% glycine and 0.5 wt% glucose at 30 °C. After overnight incubation, they were centrifuged (6000 g, 20 min) and washed with 400 ml 17 wt% (0.5 M) sucrose, 10 wt% glycerol (4 °C), spun down and resuspended in 200 ml 17 wt% (0.5 M) sucrose, 10 wt% glycerol + 50 mM EDTA (4 °C). After incubating on ice for 15 min and spinning down (6000 g, 10 min), the cells were washed again as described above and resuspended in 4 ml 17 wt% (0.5 M) sucrose, 10 wt % glycerol (4 °C). Then, 40 µl of the cell solution with 1 µl DNA (100 ng · µl<sup>-1</sup> in Tris-Buffer) was added to a chilled cuvette and pulsed using a Bio-rad Genepulser (Bio-rad, CA, USA) for 5.7 ms (2000 V, 25 µF, 200Ω), after which 1 ml M17 broth with 17 wt% (0.5 M) sucrose, 2.5 wt% glycine, 0.5 wt% glucose, 20 mM MgCl<sub>2</sub> and 2 mM CaCl<sub>2</sub> was added. The solution was added to M17 agar plates containing 0.5 wt% lactose. After incubation for 48 h, colonies were picked and restreaked.

### 3.4.6 Growth rate determination

To check the phenotype of *L. cremoris* MG1363\_GFP Lac<sup>+</sup>, we tested whether the strain was fluorescent, and whether the growth rate was similar to the growth rate on lactose of *L. cremoris* MG5267, a lactose-positive *L. cremoris* strain, and similar on glucose to its ancestor strain MG1363. Fluorescence of the strain was confirmed using flow cytometry (Accuri C6, BD, NJ, USA). To prepare the cells for inoculation for the growth rate determination, MG1363\_GFP Lac<sup>+</sup> was precultured in CDMpc on both glucose and lactose, and MG5267 and MG1363 were precultured as described above. After 16 h, the OD<sub>660</sub> was determined using a Jenway 7200 Spectrophotometer (Cole-Palmer, Stone, UK) and the cells were inoculated in duplicates in 30 ml of the same medium as in the preculture at an OD<sub>660</sub> of 0.01. To determine the growth rate, the OD<sub>660</sub> was measured every hour (Supplementary Figure 5).

### 3.4.7 Co-cultivation on plates

*L. cremoris* MG1363\_GFP Lac<sup>+</sup>, NZ9000 Glc<sup>-</sup> Lac<sup>+</sup> and MG610 were precultured as described above, spun down (8000 g, 15 min), washed in sterile PBS and resuspended in PBS to a final concentration of  $3 \times 10^7$  cells ml<sup>-1</sup>, as determined by flow cytometry (Accuri C6, BD, NJ, USA). To make the cell mixtures, cells were added together to the indicated cell concentration and strain ratio. Of the cell mixtures, 100 µl was added to plates containing CDMpc<sub>cas</sub>. The plates were incubated for 90 h at 30 °C. Afterwards, the cells were removed from the plate by adding 2 ml sterile PBS and spreading it using a sterile spreader, as described in [46]. The cell suspension was removed from the plate, and the cell concentration and cheater abundance was determined using flow cytometry (Accuri C6, BD, NJ, USA). The final cell concentration was then used to calculate a total final cell count. Together with the known initial cell count, this was used to calculate the number of times the cells had doubled using the following formula:

$$d = \log_2 \left( \frac{N_f}{N_i} \right)$$

Here,  $d$  is the number of doublings, and  $N_f$  and  $N_i$  are the final and initial cell counts, respectively. To selectively lower the growth rate of cooperator A, erythromycin was added to the agar plates at the indicated concentrations.

## Acknowledgments

We thank Sagarika Bangalore Govindaraju for her support with the experiments. We thank Felix Frey for reviewing the manuscript.

## Author contributions

Rachel Los (Conceptualization, Methodology, Software, Investigation, Data curation, Formal analysis, Writing—original draft), Tobias Fecker (Methodology, Investigation, Data curation, Formal analysis, Writing—review & editing), P.A.M. van Touw (Investigation, Data curation, Formal analysis, Writing—review & editing), Rinke J. van Tatenhove-Pel (Methodology, Resources, Supervision, Writing—review & editing), and Timon Idema (Conceptualization, Methodology, Supervision, Writing—review & editing).

## Supplementary Files

### S3.1 Supplemental methods

#### Individual-based simulation details

Each particle is modelled as a spherocylinder that consists of two spheres of diameter,  $D$ , connected by a spring with a rest length  $L$  (Figure 3.1a). This rest length is incrementally increased with growth rate  $\mu$  until it reaches the division length  $L_d$ . The particle then splits up into two daughter particles. Here, a tiny bit of noise is added in the orientation to make sure that the particles don't grow in a perfect line. For each particle pair that is within interaction range,  $R_{int}$ , of each other, the repulsive or attractive force is determined. First, the shortest distance between the particles is determined. If it is negative, i.e. there is overlap between the particles or the surface, the repulsive force is calculated as a spring force with spring constant  $k_r$ , scaling with the amount of overlap  $d$ ,

$$F(d) = -k_r \cdot d$$

This force is then distributed along the backbone inversely proportional to where along the backbone the overlap occurred. Particles also experience an attractive force between each other and with the surface. The magnitude of the particle adhesion force depends on the distance  $r$  between the centers of the particles and their relative orientations in the following way:

$$F(r, \theta, \phi) = F_{PP} \cdot \left( 4 \left( \frac{0.95D}{r} \right)^5 - 4 \left( \frac{0.95D}{r} \right)^9 \right) \cdot \cos \theta \cdot \cos \phi, \text{ for } r > D$$

where  $D$  is the diameter of the particles,  $F_{pp}$  determines the amplitude of the attractive forces, and  $\theta$  and  $\phi$  are the angles between the backbones of the two particles. The resulting force is then distributed over the two end-points of the particle inversely related to where the closest point between the particles is situated along the length of the particle. The attractive force to the surface is implemented similarly, but  $r$  is now the distance from the center of the particle to the surface.

$$F(r, \theta) = F_{PS} \cdot \left( 4 \left( \frac{0.95R}{r} \right)^5 - 4 \left( \frac{0.95R}{r} \right)^9 \right) \cdot \cos \theta, \text{ for } r > R$$

where  $R$  is the radius of the particles or half the diameter, and  $\theta$  is the angle the particle makes with the surface. Again, the force is distributed over the two spheres that make up the particle, inversely proportional to the distance of each end point to the surface. Consequently, the particles experience both a force and a torque due to the attractive and repulsive interactions. Every time step all the forces that the particles undergo are calculated and they are moved accordingly. We grow all the particles once every 5 time steps. We grow the system to a set amount of particles and then count how many of those particles are cheaters. Values for all the parameters used can be found in Supplementary Table S3.1.

**Supplementary Table S3.1: Explanation and values used for the simulation parameters**

| Parameter | Description                                                        | Value                                         |
|-----------|--------------------------------------------------------------------|-----------------------------------------------|
| $k_{int}$ | Spring constant of the internal spring                             | $0.1 \text{ Nm}^{-1}$                         |
| $D$       | Particle diameter                                                  | $1 \mu\text{m}$                               |
| $L_d$     | Length of the spring at division                                   | $4 \mu\text{m}$                               |
| $k_r$     | Spring constant of the overlap potential                           | $0.2 \text{ Nm}^{-1}$                         |
| $F_{PS}$  | Particle-surface adhesion prefactor                                | $10^{-5} \text{ N}$                           |
| $F_{PP}$  | Particle-particle adhesion prefactor                               | $10^{-5} \text{ N}$                           |
| $DT$      | Division time for a particle growing with growth rate $\mu$        | $10^5 t_s$                                    |
| $R_{int}$ | Interaction range for which mechanical interactions are calculated | $2 \cdot L_d$                                 |
| $\mu$     | Background growth rate for a cheater particle                      | $0.0002 \cdot \mu\text{m} (5 \cdot t_s)^{-1}$ |
| $K_S$     | Hill coefficient for the growth rate adjustment                    | 2.5                                           |

### Well-mixed model of costly cooperation

In order to show the effect of spatial structure on our simulated consortium, we compare it to a well-mixed system. For this we solve the following system of equations for the evolution of populations of interacting species A, B and C:

$$\frac{dA}{dt} = (1 - c + H(B, N, b))A$$

$$\frac{dB}{dt} = (1 - c + H(A, N, b))B$$

$$\frac{dC}{dt} = (1 + H(\min(A, B), N, b))C$$

Here A, B, and C are the size of the population of the corresponding strain. N is the total size of the population. The growth of the populations depends on c, which is the cost of cooperation, and H, which calculates the benefit depending on the ratio of the cooperative species as follows:

$$H(n, N, b) = \frac{\frac{n}{N} \cdot \bar{n}}{K_s + \frac{n}{N} \cdot \bar{n}} \cdot b$$

where n is the amount of beneficial particles in the total population and N is the size of the total population. Additionally, b is the benefit of cooperation,  $K_s$  is the Hill coefficient which is set to 2.5, and  $\bar{n}$  is the average number of neighbors a particle has, which is set to 7.5.

The initial conditions are the same as for the simulations, so 10 of each strain, the cheater fraction is measured when a population size of  $10^4$  particles is reached. The results are summarized in Supplementary Figure S3.2.

### A:B:C ratios and how they relate to effective distance between cooperators

If we look from the perspective of a B particle on a plate, the amount of particles that we find within radius, r, away from that particle follows a Poisson distribution,

$$P(k) = \frac{\lambda^k \cdot e^{-\lambda}}{k!} \text{ where } \lambda = \pi r^2$$

Here, k is the amount of particles and  $\lambda$  is the expectation value of the amount of particles in a circle of radius r, based on the density of the particles  $\sigma$ . Because we are interested in what the first encounter will be, we want to compare the radii for the first encounter with an A particle and the one with a C particle. The probability of having at least one A particle within a radius, r, is given by,

$$\begin{aligned} P(A) &= 1 - P(0), \\ &= 1 - e^{-\pi r^2 \sigma_A}. \end{aligned}$$

From this we can get a probability density function,

$$f(r) = \frac{d}{dr}P(A) = 2\pi r \sigma_A \cdot e^{-\pi r^2 \sigma_A},$$

which we can then use to find an expectation value of what the distance is that a B will encounter its first neighbor of type A.

$$\langle r_{AB} \rangle = \int_0^\infty r \cdot 2\pi r \sigma_A \cdot e^{-\pi r^2 \sigma_A} dr = \frac{1}{2\sqrt{\sigma_A}}.$$

Similarly, the first neighbor of type C is expected to be found at,

$$\langle r_{AB} \rangle = \frac{1}{2\sqrt{\sigma_A}}$$

When we subtract the two we get the expected relative distance which denotes who is expected to be further away from B, A or C:

$$\langle r_{AB} - r_{CB} \rangle = \frac{1}{2\sqrt{\sigma_A}} - \frac{1}{2\sqrt{\sigma_C}}$$

The particle density at inoculation,  $\sigma$ , can be easily calculated, as it is always the same, namely  $3 \cdot 10^6$  cells on a petri dish with a diameter of 9 cm. This gives us an overall initial particle density of

$$\sigma = \frac{3 \cdot 10^6}{\pi (4.5 \cdot 10^4)^2} = 0.00465 \mu\text{m}^{-2}$$

The densities of A and C are then simply the fractions of those particles times this overall density.  $\langle r_{AB} - r_{CB} \rangle$  is plotted for all  $[C]/[A]$  ratios used in the 10% cheater fraction experiments in Figure 3.4b.

### Growth study

Supplementary Figure S3.5 shows a representative example of how we calculated the growth rate  $\mu_{\text{max}}$ . As example, we describe the growth study of MG1363-GFP Lac<sup>+</sup> on 0.09 wt% glucose. We measured the OD<sub>660</sub> from duplicate shake-flasks hourly (Supplementary Figure S3.5a). We extracted the exponential phase by plotting the log-linear of OD<sub>660</sub> versus time and fitting a linear curve to the segment where the log(OD<sub>660</sub>) increased linearly, as determined by the least squares method

(Supplementary Figure S3.5b). We determined the goodness-of-fit for all replicate curves by  $R^2$  (Supplementary Table S3.2).

Gating strategy

Supplementary Fig. S3.6 shows the flow cytometry gating strategy for a representative example.

Step 1: Exclude noise

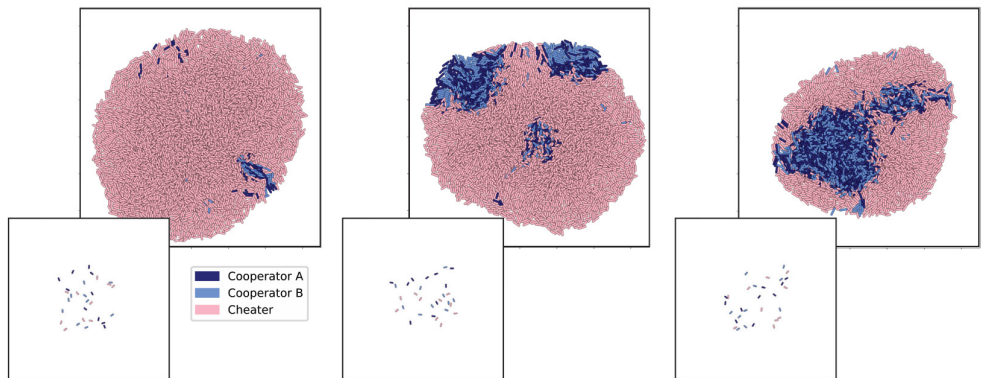
We determined the location of the background noise by the buffer by plotting the forward and side scatter of a sample consisting of only the buffer. This yielded gate 1. From a sample with cells, gate 2 containing everything except the noise was drawn.

Step 2: Determining the fraction of the fluorescent cheater cells

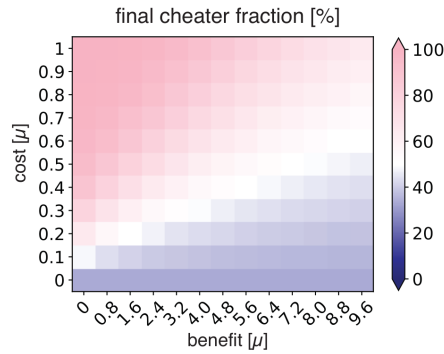
Gate 2, containing the cells, was the reference for plotting fluorescence against forward scatter. In this plot, the fluorescent cheater cells were marked as Gate 3. To calculate the cheater fraction, we divided the number of these fluorescent cells by the total cell count, excluding the noise.

Supplementary Table S3.2: Goodness-of-fit of all extracted growth curves as determined by the  $R^2$  of the  $\ln(OD_{660})$  over time and resulting  $\mu_{\max} \pm SD$  (n=2)

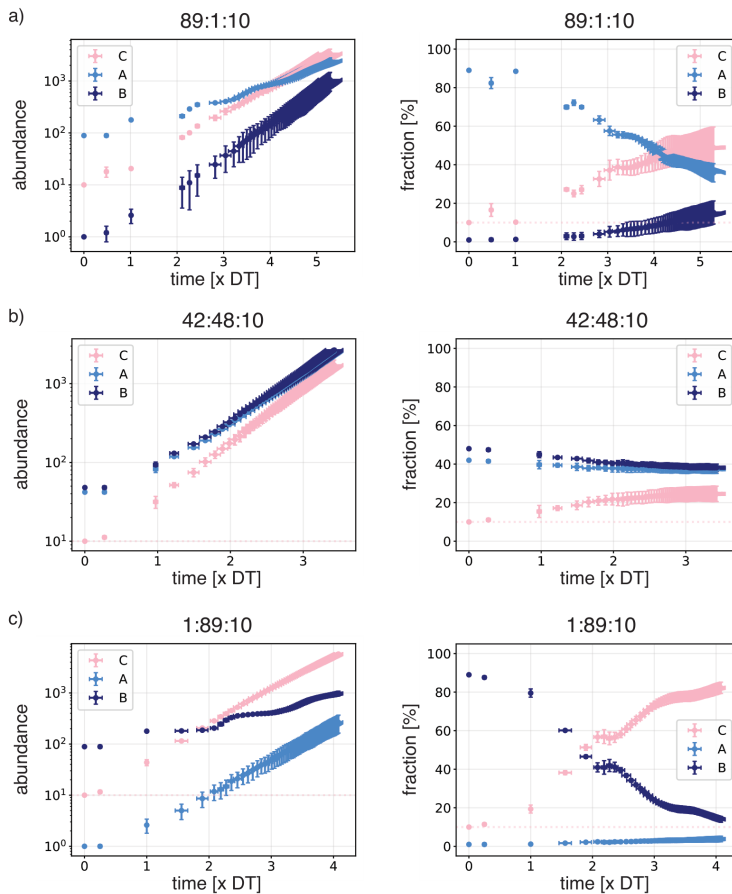
| Strain                         | Carbon Source | $R^2$ rep. 1 | $R^2$ rep. 2 | $\mu_{\max}$ (1/h) $\pm$ SD |
|--------------------------------|---------------|--------------|--------------|-----------------------------|
| MG1363_GFP Lac <sup>+</sup>    | 0.09 wt% glc  | 0.997        | 0.998        | $0.67 \pm 0.0$              |
| MG1363_GFP                     | 0.09 wt% glc  | 0.997        | 0.998        | $0.57 \pm 0.01$             |
| MG1363_GFP (Lac <sup>+</sup> ) | 0.09 wt% lac  | 0.996        | 0.999        | $0.66 \pm 0.00$             |
| MG5267                         | 0.09 wt% lac  | 0.998        | 0.996        | $0.64 \pm 0.00$             |



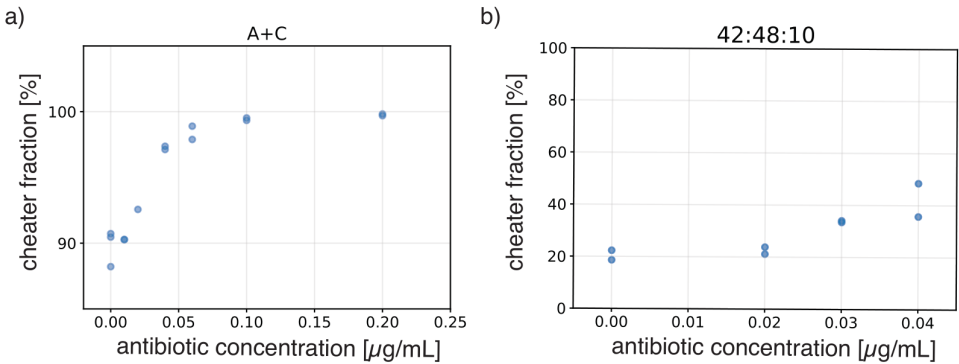
Supplementary Figure S3.1: Examples of three different final colonies for the same input parameters for the cross-feeding interaction; value for cost = 0.8 and benefit = 4.0. In the bottom-left corner of each final colony, the initial configuration of that run is shown. Area shown is always  $130 \times 130 \mu\text{m}^2$ .



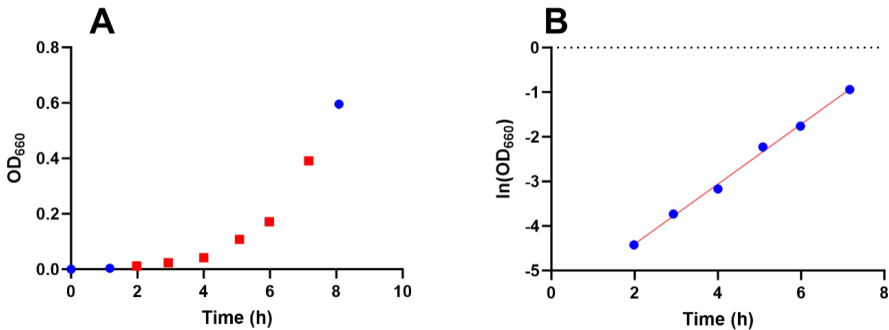
**Supplementary Figure S3.2: Final cheater fraction for a well-mixed system of A, B and C growing to a population size of  $10^4$ . Interactions are implemented similarly to the IBM, see SI.**



**Supplementary Figure S3.3: Abundance and fraction of the different particle types over simulation time for the simulation data points of Figure 5b. A:B:C ratios a) 89:1:10, b) 42:48:10, and c) 1:89:10. All points are averages and standard deviations of 5 runs.**

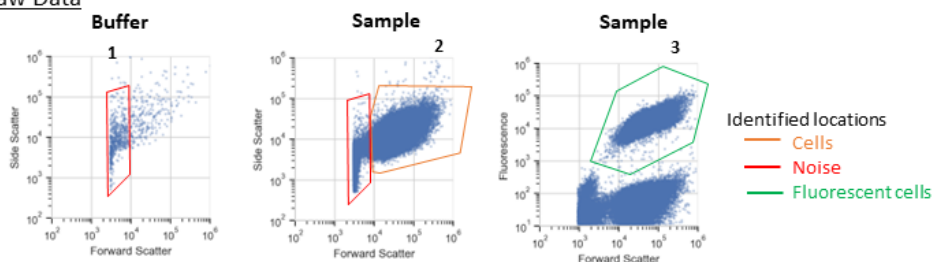


**Supplementary Figure S3.4: Final cheater fraction for different concentrations of erythromycin** a) in plates containing casein and amino acids inoculated with A & C together, and b) in plates containing casein inoculated with A, B & C in a 42:48:10 ratio.

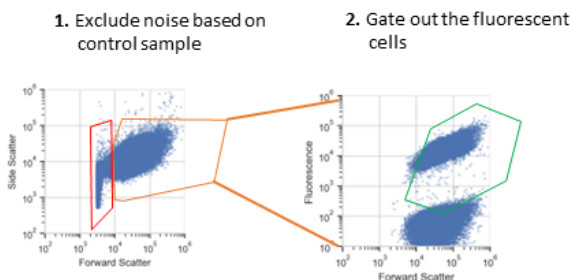


**Supplementary Figure S3.5: Growth curve of MG1363-GFP Lac<sup>+</sup> (cheater).** a) OD<sub>660</sub> over time. The data points used to calculate the exponential phase are marked with red squares. b) ln(OD<sub>660</sub>) of the exponential phase measurements over time. A linear curve was fit to the measurements by least-squares fit.

### Raw Data



### Determination of number of fluorescent cheater cells



**Supplementary Figure S3.6: Flow cytometry gating strategy.** Areas within the forward scatter – side scatter and fluorescence – forward scatter were identified using control samples. The gating excluded noise and was able to clearly distinguish between fluorescent and nonfluorescent cells to calculate the %cheater fraction.

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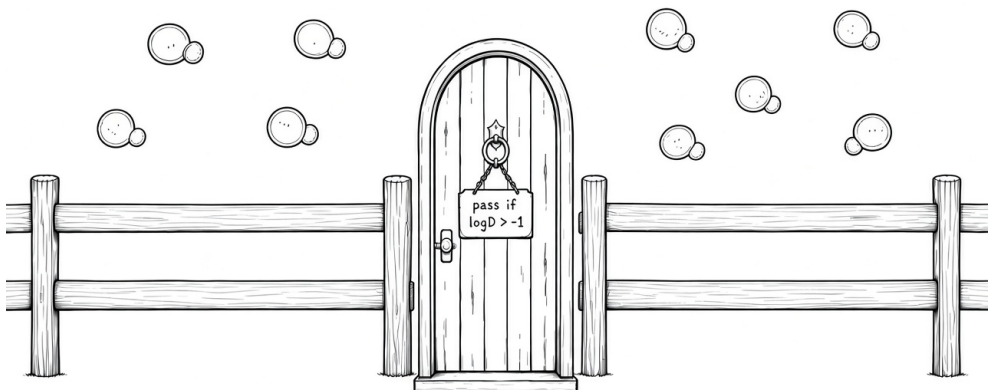
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It is wrong to think that the task  
of physics is to find out how  
nature is.

Physics concerns what we can  
say about nature.

Niels Bohr



# Chapter 4

## **The effect of substrate and product transfer between polydisperse microdroplets on biological high-throughput screens**

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Essentially as published in Scientific Reports (2026) 16:4318 1-12  
<https://doi.org/10.1038/s41598-025-25878-5>

## Abstract

4 Microdroplet encapsulation of cells is a powerful tool for high-throughput biological testing. The technique is widely used in mutant selection and strain engineering, where strict compartmentalization is often required to prevent transfer of cells or molecules between droplets. While molecular transfer has been reported for fluorophores, the potential transfer of common small substrates and products, and its impact on biological outcomes, has not been elucidated. Here, using a biosensor system, we demonstrate that biologically relevant molecules such as small alcohols (e.g. ethanol) and weak acids can transfer between droplets. The transfer can alter carbon availability and pH in neighboring droplets. Ethanol, a common product in biological systems, partitions directly into the oil phase, leading to unintended cell growth in adjacent droplets. Weak acids also transfer, decreasing the pH in neighboring droplets. Our results show that the transfer of small, hydrophobic molecules, commonly found in biological systems, can significantly alter experimental outcome. These findings highlight the need for validating droplet compartmentalization in assays involving small, amphiphilic molecules and careful selection of substrates that are not likely to transfer among droplets.

## 4.1 Introduction

Over the last decade, encapsulation of cells in the water droplets of a water-in-oil emulsion has emerged as a powerful tool for meeting the growing demand for high-throughput biological experimentation. Unlike other high-throughput methods like fluorescence-assisted cell sorting (FACS), which require the desired product to be intracellular, droplet encapsulation directly links the producer cell to its environment, making it possible to screen cells with secreted metabolites [1].

Traditionally, microtiter plate assays have been used for similar screening applications, but their throughput is restricted by plate capacity and liquid handling. In contrast, droplet encapsulation enables cost-effective screening of large parameter spaces, such as mutant libraries, with volumes ranging from femto- to nanolitres [2,3]. This technique has been widely applied in laboratory studies, for example to screen strain libraries to identify variants with elevated protein production [4], enhanced metabolite secretion [5], or improved biomass yield [6].

A key feature of microdroplet systems is their ability to keep the compartments isolated, preventing transfer of substrates or metabolites into neighbouring droplets. To achieve this, researchers mix the aqueous phase containing cells and nutrients with an oil phase, forming an emulsion, stabilized by a surfactant [7]. Polydisperse emulsions (broad size distribution) are easily generated via vortexing or homogenization [8], while microfluidic chips produce more uniform, monodisperse emulsions [9]. Earlier systems used hydrocarbon-based oils and surfactants, which limited oxygen solubility and allowed for some organic molecules, like ethanol or fluorescein, to transfer into the oil phase [10–12]. To address these issues, fluorinated oil/surfactant systems (using for example HFE7500 as oil phase) have become increasingly popular. Here, most carbon-hydrogen bonds of the oil phase and surfactant are replaced by carbon-fluoride bonds, which reduces interactions with both hydrophilic and hydrophobic molecule groups [10,13].

Although fluorinated emulsions improve molecule retention in the droplets, recent studies suggest that certain classes of molecules, particularly large, hydrophobic aromatics like fluorophores, can transfer between droplets [14–17]. The molecules transfer at a rate proportional to their hydrophobicity [16], which is unexpected since organic molecules do not mix well with fluorinated liquids [18]. Since fluorophores often serve as experimental readout, their transfer can compromise experimental accuracy [19]. This issue has prompted researchers to investigate the underlying transfer mechanism [19,20]. Rather than directly diffusing to the neighbouring droplet via the oil phase, these large molecules are often shuttled via micelles formed by excess surfactant molecules, which display an organic head group [17,20,21]. These findings recently sparked an extensive survey of different molecule classes

and their ability to transfer between droplets [14].

Beyond fluorophores, biological microdroplet experiments typically require the strict compartmentalization of small molecules, such as substrates or products. For example, when selecting for high-yield mutants, the available carbon source per cell must be 'privatized' in the droplet [6]. However, common metabolic byproducts of biological culturing, such as ethanol, have been suspected to transfer in fluorinated systems [22]. Such transfer can disrupt the strict compartmentalization with potential impact for the neighbouring droplets. For example, ethanol leakage could inhibit growth or provide extra carbon for cells in neighbouring droplets, skewing screening experiments or strain optimization for bioethanol production. For some common molecule classes like amines or organic acids, parameters (e.g., molecule size, hydrophobicity) have been established to assess their potential to transfer [14]. Still, the field lacks a predictive framework to assess the risk of molecule transfer particularly for molecules commonly encountered in biological experiments, covering common substrates and products.

Here, we tested a range of biologically relevant small molecules to evaluate their potential to transfer between droplets in polydisperse systems and the impact on experimental outcomes. We developed a biosensor-based assay to directly test the transfer of relevant substrates, such as sugars, amino acids and alcohols, and small products like organic acids, which can be readily adapted to test other molecules. By monitoring cell growth in adjacent droplets, we quantify the biological effect of small molecule transfer. Further, we test the effect of weak acid transfer on the pH of neighbouring droplets and give a perspective on preventing unwanted transfer. Unlike previous studies, our approach directly links molecular transfer to biological outcomes. Based on our results, we recommend substrates and products suitable for reliable use in droplet-based experiments.

## 4.2 Results

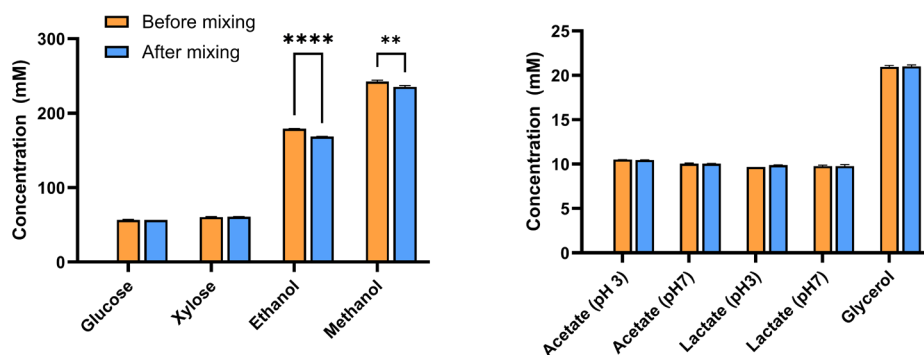
In water-in-oil (w/o) emulsions, individual cells are compartmentalized in microdroplets of 10–100  $\mu\text{m}$  diameter, enabling rapid screening [23]. The cells are separated from each other by an oil phase, typically a fluorinated oil like HFE7500. To ensure that droplets form independent compartments, neither cells nor substrates should transfer between droplets. To test to what extent commonly encountered molecules disrupt compartmentalization by transferring between droplets and how this transfer impacts compartmentalization in high-throughput droplet screens, we did a series of experiments focusing on common substrates and products.

### 4.2.1 Alcohols directly partition into HFE7500 oil

We tested whether a range of substrates and products typically encountered in

biological systems partition from the water phase into HFE7500 oil. To do so, we prepared aqueous solutions of sugars, alcohols, and weak acids and mixed them with HFE7500 oil in a 1:9 (aqueous:oil) volumetric ratio. We measured molecule concentrations using HPLC before and after mixing with the oil, to identify whether the molecule partitions into the oil phase. We chose the concentrations of each compound to maximize the signal-to-noise ratio of the HPLC, while maintaining biological relevance. For example, ethanol was chosen to be  $180 \text{ mM} = 8.3 \text{ g} \cdot \text{L}^{-1}$ . Typical concentrations for growing *S. cerevisiae* on ethanol are between  $3.5$  and  $7.5 \text{ g} \cdot \text{L}^{-1}$  [24], while aerobic fermentations by *S. cerevisiae* on  $20 \text{ g} \cdot \text{L}^{-1}$  glucose typically yields  $7.5 \text{ g/L}$  ethanol [25], close to the chosen value. Similarly,  $0.2 - 0.4 \text{ g} \cdot \text{L}^{-1}$  acetic acid is produced by *S. cerevisiae* on  $20 \text{ g} \cdot \text{L}^{-1}$  glucose, close to the chosen tested concentration ( $10 \text{ mM} = 0.6 \text{ g} \cdot \text{L}^{-1}$ ) [26].

While none of the sugars and weak acids showed any loss in concentration, the concentration of the alcohols ethanol and methanol dropped significantly after mixing with HFE7500 oil ( $6.3 \pm 0.3 \%$  and  $3.0 \pm 0.54 \%$ , respectively, Figure 4.1). This indicates direct partitioning of alcohols into the oil phase, potentially disrupting the compartmentalization of biological systems in microdroplet experiments.



**Figure 4.1: Partitioning of common substrates and products into HFE7500 oil.** The concentration of the prepared solution is measured before and after mixing with HFE7500 to give an estimation how much of each molecule partitioned into the oil phase. The bars represent the mean of three replicates  $\pm$  SD. Asterisks indicate statistically significant differences ( $\alpha=0.01$ ) (\*\*:  $p = 0.0033$ , \*\*\*\*:  $p = 0.000006$ ; unpaired, one-tailed Student's t-test). (a) Sugars and alcohols. (b) Weak acids and glycerol. The figure was split for clarity.

#### 4.2.2 Ethanol transfer between microdroplets enables growth of *Saccharomyces cerevisiae* cells

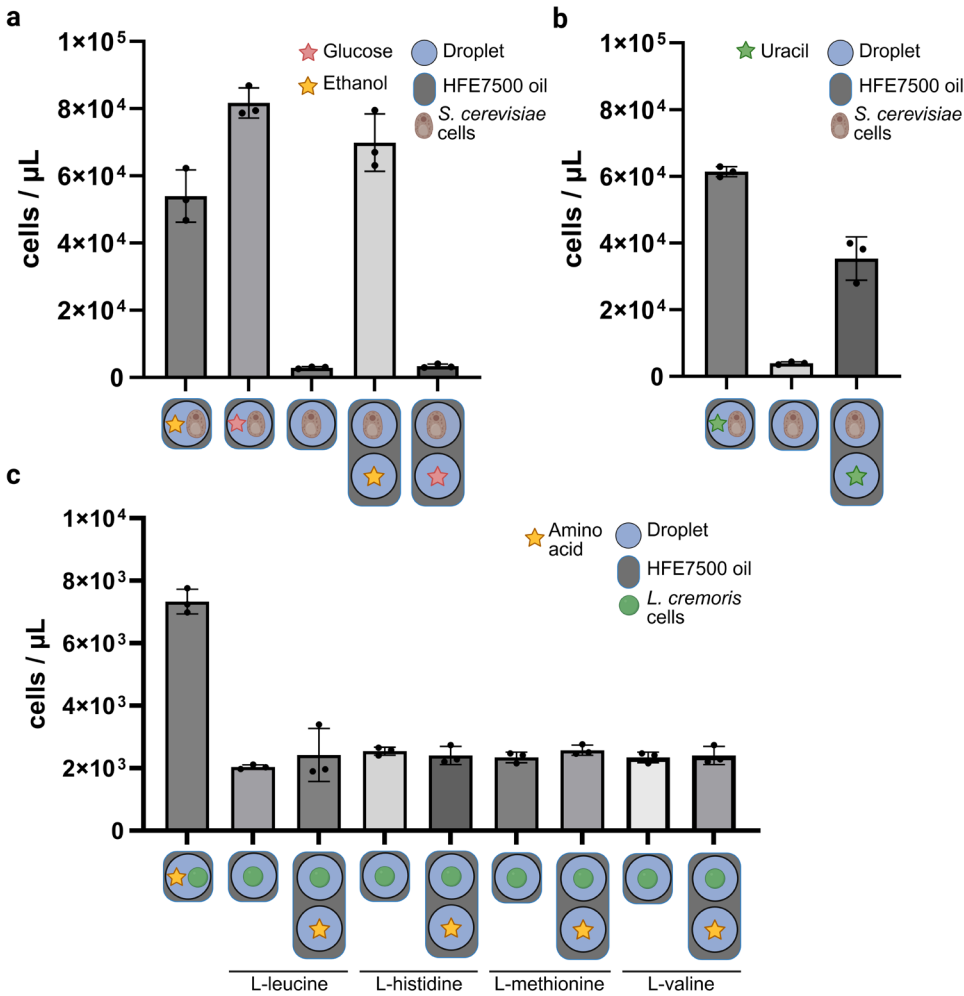
To assess the impact of ethanol partitioning on microdroplet compartmentalization, we investigated whether ethanol transfer between droplets could allow for growth of *S. cerevisiae* cells. Polydisperse droplets with a representative volume  $\pm$  SEM of  $37.6 \pm 6.9$  pL were generated using vortexing (Supplementary section S4.1, Supplementary Figure S4.1). *Saccharomyces cerevisiae* was selected for its ability to grow aerobically on both glucose and ethanol.

We first confirmed that *S. cerevisiae* could grow in microdroplets containing ethanol or glucose, and could not grow in droplets without a carbon source (Figure 4.2). To test for carbon source transfer between droplets, we mixed two emulsions – one with cells only and the other one with carbon source only. The results showed restored cell growth in the mixed emulsions containing ethanol, but not in those containing glucose (Figure 4.2). This observation indicates that ethanol, unlike glucose, can transfer between microdroplets and induce cell growth.

The emulsions generated containing either glucose or ethanol did not differ significantly in size and size distribution in time (Supplementary Figure S4.2), indicating that any selective transfer of either substrate could not be explained by differences in microdroplet generation. Additionally, the amount of small droplets ( $< 2\mu\text{m}$ ) produced during emulsification and potentially acting as molecular shuttles, did not differ significantly between either emulsion (Supplementary Figure S4.2), suggesting that the chosen substrate did not impact the droplet generation process.

Next, we tested if other molecule classes would transfer, enabling growth in neighbouring droplets. In this regard, we tested four amino acids (L-leucine, L-valine, L-histidine, and L-methionine), as well as the nucleotide uracil with auxotrophic strains. We chose *L. cremoris* MG1363 as biosensor, as it is auxotrophic for these amino acids [27], and *S. cerevisiae* CEN.PK113-5D as uracil auxotroph [28].

While none of the amino acids showed transfer that enabled growth of the auxotrophic *L. cremoris* strain (Figure 4.2c), the nucleotide uracil transferred sufficiently to enable growth of the *S. cerevisiae* strain (Figure 4.2b).



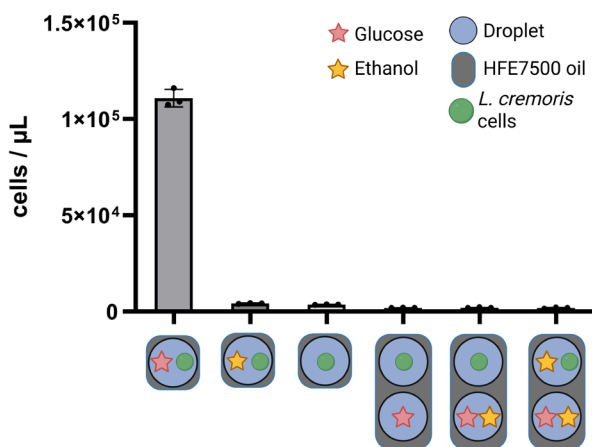
**Figure 4.2: Testing the biological effect of molecule transfer in microdroplets.** a) Molecular transfer of glucose (red stars) and ethanol (yellow stars) in HFE7500 oil. The *S. cerevisiae* cells were cultured in droplets containing 2 wt% ethanol, 8 wt% glucose or no carbon source, in which case the droplets were mixed with other droplets containing these substrates. Cell growth after 72h was assessed by breaking the emulsion and subsequent flow cytometry. n=3 mean  $\pm$  SD. b) Molecular transfer of uracil in HFE7500 oil. The auxotrophic *S. cerevisiae* cells were cultured in droplets containing 8 wt% glucose but lacking uracil and mixed with an emulsion containing uracil. Cell growth after 72 h was assessed by breaking the emulsion and subsequent flow cytometry. n=3 mean  $\pm$  SD c) Molecular transfer of a selection of amino acids. Auxotrophic *L. cremoris* cells were grown in four different emulsions, each missing one amino acid and mixed with emulsions containing these amino acids. Cell growth after 72 h was assessed by breaking the emulsions and subsequent flow cytometry n=3 mean  $\pm$  SD. Figure was created in BioRender. Fecker, T. (2025); <https://BioRender.com/bc2n7rs>

### 4.2.3 Ethanol does not induce a transfer of glucose between microdroplets

In biological experiments with fermenting organisms like *S. cerevisiae*, ethanol is a common product when culturing on glucose. Due to its amphiphilic nature, ethanol has been observed to act as co-solvent, potentially inducing the transfer of molecules by weakening the droplet/oil interface [29]. Co-transfer of ethanol together with glucose would therefore impact strict compartmentalization of glucose.

To investigate whether ethanol could act as a co-solvent for glucose transfer in microdroplets, we employed a system similar as before using *Lactococcus cremoris* as biosensor. The *L. cremoris* strain was chosen for its ability to consume glucose but not ethanol, making it an ideal indicator for glucose transfer in the presence of ethanol. We again prepared two types of emulsions: one containing cells but no carbon source, and another containing either glucose alone or glucose with ethanol. After mixing the emulsions, we incubated the emulsions for 48 h and assessed bacterial growth.

Our results showed no restoration of growth of *L. cremoris* after the 2-day incubation period, regardless of the presence or absence of ethanol in the adjacent droplets, indicating that ethanol does not act as a co-solvent for glucose in this system (Figure 4.3).

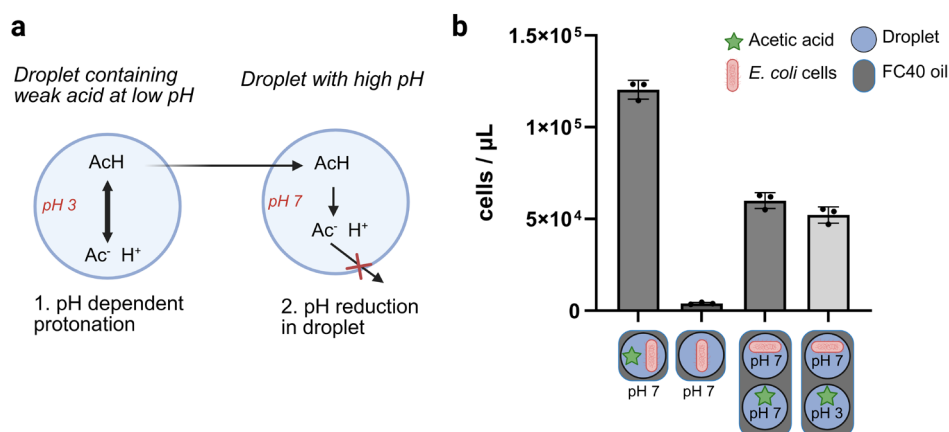


**Figure 4.3: Ethanol as a co-solvent for glucose.** Two types of emulsions were generated, containing CDMpc medium with either 2 wt% ethanol or 0.09 wt% glucose or no carbon source. *Lactococcus cremoris* was used as biosensor for molecule transfer. Since the organism can only grow on glucose, growth would indicate glucose transfer in the presence of ethanol. Growth was measured after 48 h incubation using flow cytometry.  $n=3$ , mean  $\pm$  SD. Figure was created in BioRender. Fecker, T. (2025): <https://BioRender.com/iol885d>

## 4.2.4 Weak acids transfer and show uncoupling between droplets

To investigate the transfer of a third class of common molecules in biological experiments, weak acids, we selected acetic acid, a common metabolic product and carbon source. Since this acid shows pH-dependent protonation, it may also show differences in transfer between droplets, depending on the droplet pH (Figure 4.4a).

To test a pH-dependent transfer of acetic acid, we used acetic acid-consuming *Escherichia coli* cells as biosensors. We prepared emulsions containing *E. coli* cells and mixed them with emulsions containing droplets with 0.1 wt% acetate at pH 3 or pH 7, at which the acid is either mostly protonated or deprotonated, respectively ( $pK_a = 4.76$ ). After 48 h of incubation, the cell concentration had increased irrespective of the pH of the acid-containing droplets (Figure 4.4b), suggesting that, in emulsions, acetic acid can transfer between droplets, even at a pH at which it is mostly deprotonated.



**Figure 4.4: Transfer of acetic acid between droplets.** a) Schematics of weak acid decoupling. The weak acid is mostly protonated if the surrounding pH is below the  $pK_a$ , which increases its hydrophobicity enabling it to pass through membranes. Once in the higher pH compartment, it deprotonates, reducing the pH inside the compartment. b) Two types of emulsions were generated, containing M9 medium with either 0.1 wt% acetic acid or no carbon source, using *E. coli* as biosensor for molecule transfer. The organism can grow on acetic acid, so growth indicates the transfer of acetic acid between two droplets.  $N=3$ , mean  $\pm$  SD. Figure created in BioRender. Fecker, T (2025): <https://BioRender.com/q670x9x>

We hypothesized that acetic acid would preferentially transfer in the protonated form due to its higher hydrophobicity [30] and then lower the pH in the droplets containing cells, resulting in a net transfer of protons among the droplets (Figure 4a). To test this, we added the pH-dependent fluorophore 5,6-carboxyfluorescein to the emulsions, which exhibits green fluorescence above pH 5 [31]. Using fluorescence

microscopy and an image analysis pipeline (described in supplementary files, section S4.5), we quantified the fluorescence intensity of individual droplets. As expected, droplets containing buffer at pH 7 exhibited strong green fluorescence, while those at pH 3 showed no fluorescence (Figure 4.5a, Supplementary Figure S4.3).

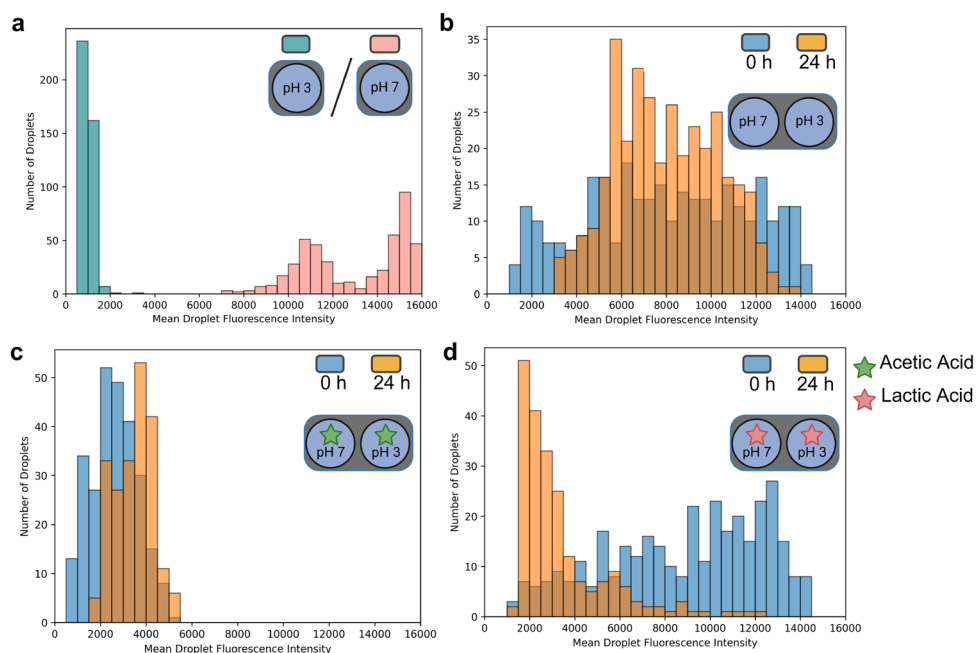
Next, we mixed emulsions of droplets at pH 7 with droplets at pH 3 and measured fluorescence immediately and after 24 hours. In the absence of weak acids, both droplet populations remained distinct over time, indicating stable pH conditions (Figure 4.5b, Supplementary Figure S4.3). We observed a minor fluorescence shift in this system, suggesting slow pH equilibration, potentially via buffer components present in the medium.

When adding 0.4 wt% acetic acid to the pH 3 droplets, we observed an immediate decrease of fluorescence of the pH 7 droplets, which persisted after 24 h. This change indicated rapid transfer of protons facilitated by acetic acid (Figure 4.5c). In contrast, the mixture with 0.4 wt% lactic acid initially maintained two distinct populations before converging to a single, low-intensity population after 24 h, suggesting slower transfer of lactate (Figure 4.5d, Supplementary Figure S4.3). These results demonstrate that weak acids can influence the pH of neighboring droplets in the system, potentially impacting the growth of cells in neighboring droplets.

#### 4.2.5 Influence of Oil Type and Surfactant on Ethanol Transfer

To investigate if the transfer of ethanol among microdroplets could be reduced by selecting another oil, we compared ethanol partitioning in FC40, HFE7500 and mineral oil. Unlike HFE7500, which contains a hydroxyl group, FC40 is perfluorinated, meaning that all CH groups are replaced by CF groups. The hydroxyl group in HFE7500 may interact with the hydroxyl group of ethanol, facilitating its solubility in the oil and thereby accelerating its transfer to other microdroplets. To test this, we mixed aqueous ethanol solutions with all three oils and measured the resulting ethanol partitioning in comparison to the water phase without oil. We chose a lower ethanol concentration than in Figure 4.1 to reflect the range of ethanol concentrations found in biological experiments [24,25]. Despite being perfluorinated, ethanol still showed significant partitioning into FC40, however less than into HFE7500 (Figure 4.6a). Mineral oil showed the highest ethanol partitioning of all tested oils, and it is likely that the biological outcome would therefore be similar or exacerbated compared to HFE7500.

To assess if the reduced ethanol partitioning into FC40 would be sufficient to prevent growth via ethanol transfer, we repeated the biosensor assay using FC40 as the oil phase. Similar to the HFE7500 experiment (Figure 4.2), droplets containing *S. cerevisiae* cells were mixed with droplets containing a carbon source. When ethanol was present, cells grew, indicating ethanol transfer between droplets, while



**Figure 4.5: Quantification of droplet fluorescence intensity to assess weak-acid-mediated pH changes.** Droplet fluorescence serves as a proxy for internal pH, based on the pH-sensitive emission of fluorophore 5,6-carboxyfluorescein. Each histogram represents the distribution of mean fluorescence intensities of droplets across several fluorescence microscopy images. a) Fluorescence distribution of droplets containing M9 medium with 0.4 wt% acetate adjusted to either pH 3 or pH 7. b-d) Mixed emulsions containing droplets at pH 3 and pH 7, with or without added weak acids, were imaged immediately after mixing ( $t = 0$  h) and after 24 h ( $t = 24$  h). pH was adjusted to ensure mostly full protonation or deprotonation of weak acids. b) Mixtures of droplets without added acids at pH 3 and pH 7 (M9 only). c) Mixtures of droplets containing 0.4 wt% acetate at pH 3 and pH 7. d) Mixtures of droplets containing 0.4 wt% lactate at pH 3 and pH 7. Figure created in BioRender. Fecker, T. (2025): <https://BioRender.com/ciaxng7>

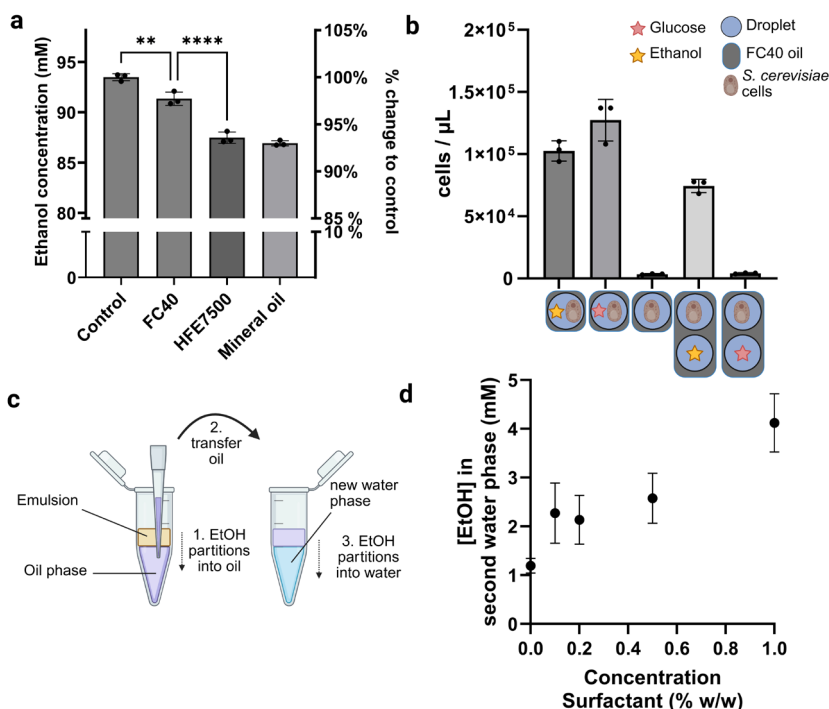
no growth was observed when glucose was used, confirming no glucose transfer (Figure 4.6b). The results are consistent with those obtained with HFE7500, suggesting that using FC40 as the oil phase could not impede transfer-mediated growth despite its reduced ethanol partitioning.

We hypothesized that the ethanol transfer between microdroplets by direct partitioning into the oil phase would be enhanced in the presence of surfactant. Surfactants are required to stabilize the emulsion but have been shown to increase the solubility of small molecules in the oil phase and therefore exacerbate the transfer.

To test this, we generated emulsions containing ethanol in the aqueous phase with

varying surfactant concentrations in the oil. After phase separation, we recovered the oil phase from the bottom and added it to a second water phase. Subsequently, we measured the ethanol concentration using HPLC after allowing the ethanol in the oil phase to partition into the water phase for 6 h (Figure 4.6c). Results showed that the surfactant had a measurable influence on increasing ethanol partitioning (Figure 4.6d).

These findings suggest that changing the oil or adjusting the surfactant concentration may not be sufficient to prevent ethanol transfer in microdroplet experiments, highlighting the challenges in maintaining compartmentalization in such systems.



**Figure 4.6: Effect of oil choice and surfactant on the transfer of ethanol.** a) Reduction of ethanol concentration in an aqueous phase when mixed with different oils at 1:9 water: oil ratio (avg  $\pm$  SD,  $n=3$ ). Statistical test: multiple t.tests, unpaired, corrected with Holm-Šidák method (\*\*:  $p=0.0012$ , \*\*\*\*= $p<0.0001$ ). b) Transfer of ethanol in a biological experiment using the perfluorinated FC40 oil. Cell growth indicated ethanol transfer and a disruption of compartmentalization (avg  $\pm$  SD,  $n=3$ ). c) Experimental scheme to assess the partition coefficient of ethanol in oil. An emulsion is generated using a water phase (ethanol in  $\text{dH}_2\text{O}$ ) and an oil phase (HFE7500 with surfactant). After phase separation, the oil phase, now containing ethanol, is transferred to a fresh water phase. Ethanol partitions into the new water phase, and its concentration is quantified by HPLC. Figure created in BioRender. Fecker, T. d) Ethanol concentration in secondary water phase with varying surfactant concentration, as described in c) (avg  $\pm$  SD,  $n=3$ ). Figure created in BioRender. Fecker, T. (2025): <https://BioRender.com/2b2d7pt>

## 4.3 Discussion

We found that several molecules commonly used in biological high-throughput experiments can transfer between droplets, disrupting the intended compartmentalization. Most notably, short chain alcohols and weak acids were able to transfer, leading to unintended cell growth and pH changes in neighbouring droplets. Ethanol was able to directly partition into the oil phase across different oils and concentrations. The extent of partitioning into HFE7500 oil was comparable for two different concentrations ( $6.3 \pm 0.3$  % in Fig. 4.1 versus  $6.2 \pm 1.2$  % in Figure 4.6a), supporting the robustness of this phenomenon across a biologically relevant concentration range. In contrast, sugars like glucose did not transfer, as indicated by the absence of cell growth in other droplets. While previous studies have reported molecule transfer in fluorinated emulsions[14,16], our results demonstrate the functional consequences for biological droplet assays in polydisperse systems.

The transfer behaviour of different molecules between droplets can be in part explained by their physicochemical properties. Short-chain alcohols such as ethanol are small, amphiphilic molecules with high water/octanol partition coefficients, allowing for transfer ( $\log P$ , see Table 4.1). In comparison, sugars as well as amino acids, which have a low  $\log P$  due to their zwitterion status at physiological pH, did not transfer, while the nucleotide uracil with a  $\log P$  of -1.1 (table 4.1), which is close to the  $\log P$  of methanol, did transfer. Although  $\log P$  is defined for hydrocarbon-based oils and may not directly translate to fluorinated oils [16], previous studies have found a strong correlation between molecule hydrophobicity and transfer rate in droplet systems [14,16,18,19]. In a recent survey of molecules,  $\log P$  was identified as the strongest predictor of molecule transfer [14]. Our findings are consistent with these results and support the use of hydrophobicity as a predictor of transfer susceptibility in microdroplet assays.

Weak acids, such as acetic acid, also showed inter-droplet transfer. When fully protonated, these molecules have  $\log P$  values similar to short-chain alcohols, while also being small ( $<100 \text{ g} \cdot \text{mol}^{-1}$ ) and amphiphilic (Table 4.1). We showed that protonated acetic acid could transfer from pH 3 droplets to pH 7 droplets, where it was consumed by growing organisms. This behaviour resembles weak acid uncoupling, in which protonated weak acids diffuse from a low-pH environment into cells, deprotonate in the higher-pH cytoplasm, and lower the intracellular pH [32]. Using a pH-sensitive fluorophore, we confirmed that the transfer not only resulted in a delivery of carbon to the droplets but also lowered the pH in neighbouring droplets, suggesting that droplet emulsions are susceptible to weak acid uncoupling at low pH (Figure 4.5).

When deprotonated at neutral pH, weak acids become negatively charged and

exhibit much lower partition coefficient values, comparable to glucose (Table 4.1,  $\log D$ ). Despite this, we still observed transfer and resulting growth in other droplets (Figure 4.4). There is still a fraction of protonated acetic acid at this pH, which may have transferred, causing the cell growth in the neighbouring droplets. Conversely, a similar effect has been reported in water-in-oil-in-water emulsions, where transfer of hydrophilic molecules can occur via micelles or small aqueous compartments [21]. The amphiphilic nature of weak acids possibly enables interactions with surfactant molecules, which increases their solubility in the oil phase and mediates transfer between droplets via inverse micelles [19,20].

Shuttling by inverse micelles might also exacerbate the transfer of small-chain alcohols. Such a dual mechanism has been reported for other molecules [14,33]. Consistent with this, we observed that ethanol transfer increased with rising surfactant concentration (Figure 4.6d), indicating that micelle-mediated transport may exacerbate molecule transfer for small, amphiphilic molecules. Further experiments to test the effect of reverse micelle shuttling could elucidate the contribution of the different mechanisms more clearly, potentially leading to new methods to prevent transfer.

The observed transfer of some common molecules can potentially affect biological experiments by disrupting the droplet compartmentalization, especially in setups that require precise control over nutrient availability, such as yield-based selection screens. Here, each droplet is supplied with a defined, limited amount of substrate, and cells that achieve high cell number yield per unit carbon are selected for [6,39]. If substrates or metabolic byproducts transfer between compartments, droplets containing fast-growing cells could become carbon sinks, allowing them to scavenge additional substrate from neighbouring droplets, and appearing with a higher yield [40].

As an example for setups that might be affected, Rabbers *et al.* successfully evolved *E. coli* strains with improved yield on glucose and the overflow metabolite acetate [41]. Although the concentrations of these byproducts are typically low and diluted over the system, their leakage may still affect the producer cells, which lose carbon to the surrounding environment. Microdroplet systems have also been used to select strains for improved ethanol production [42,43], a setup that could similarly be affected by inter-droplet transfer. Production of weak acids at low pH may unintentionally acidify adjacent droplets, potentially reducing cell viability.

Controlled metabolite transfer between droplets could potentially be exploited intentionally to enable new experimental designs. For example, methanol – a molecule we showed to diffuse readily into fluorinated oil (Figure 4.1) – is a well-established inducer of gene expression in methylotrophic yeasts such as *Komagataella phaffi* (*Pichia pastoris*) [44]. A mutant library could be encapsulated in droplets and

**Table 4.1: Tested molecules and selected physicochemical properties.**  $\log P$  refers to the octanol/water partition coefficient of the neutral molecular species.  $\log D$  refers to the distribution coefficient of all molecular subspecies at a certain condition, in this case at a pH of 7.4. For non-ionizable molecules,  $\log D$  is equivalent to  $\log P$ ; pH-dependent  $\log D$  values are listed here for ionizable compounds.

| Molecule      | Molecular Formula                                           | Molecular Weight (g/mol) | $\log D$ (pH 7.4) <sup>a</sup> | $\log P$ <sup>b</sup> | pK <sub>a</sub>              |
|---------------|-------------------------------------------------------------|--------------------------|--------------------------------|-----------------------|------------------------------|
| Ethanol       | C <sub>2</sub> H <sub>4</sub> OH                            | 46                       | -                              | -0.31                 | -                            |
| Methanol      | CH <sub>3</sub> OH                                          | 32                       | -                              | -0.77                 | -                            |
| Acetic acid   | C <sub>2</sub> H <sub>4</sub> O <sub>2</sub>                | 60                       | -2.9                           | -0.17                 | 4.8 [34]                     |
| Lactic acid   | C <sub>3</sub> H <sub>6</sub> O <sub>3</sub>                | 90                       | -3.7                           | -0.72                 | 3.8 [35]                     |
| Xylose        | C <sub>5</sub> H <sub>10</sub> O <sub>5</sub>               | 150                      | -                              | -2.5                  | -                            |
| Glucose       | C <sub>6</sub> H <sub>12</sub> O <sub>6</sub>               | 180                      | -                              | -3.0                  | -                            |
| Glycerol      | C <sub>3</sub> H <sub>8</sub> O <sub>3</sub>                | 92                       | -                              | -1.8                  | -                            |
| Uracil        | C <sub>4</sub> H <sub>4</sub> N <sub>2</sub> O <sub>2</sub> | 112                      | -0.87                          | -1.1                  | 9.4 [36]                     |
| Amino acids   | Molecular Formula                                           | Molecular Weight (g/mol) | $\log D$ (pH 7.4) <sup>a</sup> | $\log P$ <sup>c</sup> | pK <sub>a</sub> <sup>b</sup> |
| Leucine       | C <sub>6</sub> H <sub>13</sub> NO <sub>2</sub>              | 131                      | -1.9                           | 0.7 <sup>c</sup>      | 2.4 / 9.6                    |
| Valine        | C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub>              | 117                      | -2.2                           | 0.2 <sup>c</sup>      | 2.4 / 9.6                    |
| Aspartic acid | C <sub>6</sub> H <sub>9</sub> N <sub>3</sub> O <sub>2</sub> | 155                      | -3.1                           | -1.3                  | 1.8 / 6.0 / 9.2              |
| Histidine     | C <sub>5</sub> H <sub>11</sub> NO <sub>2</sub> S            | 149                      | -1.9                           | 0.4                   | 2.3 / 9.2                    |

<sup>a</sup> Extracted from ChEMBL [37]

<sup>b</sup> Extracted from PubChem [38]

<sup>c</sup> Calculated using ChemSpider's ACD/Labs model

grown under non-inducing conditions, after which methanol is administered into the oil phase or into reservoir droplets to diffuse into the target droplets and induce targeted gene expression.

A similar strategy has been demonstrated for inducers such as IPTG or AHL, which successfully diffused between droplets to induce GFP expression in a microfluidic setup [45]. However, this approach would require careful tuning of droplet spacing and methanol transfer rate to achieve desired delivery. More broadly, small-molecule diffusion could serve as an in situ substrate delivery system, effectively simulating a miniaturized fed-batch system within an emulsion [46]. This opens new possibilities in high-throughput screening setups that are currently static.

To mitigate unintended molecule transfer between droplets, several strategies have been previously explored, among others the use of additives to reduce molecule transfer. For example, bovine serum albumin (BSA) has been shown to interact with transferring fluorophores, retaining them in the water phase [12,17,46]. Similarly, novel surfactants have been developed and shown to reduce molecule transfer [47,48]. Another strategy replaces conventional surfactants with solid particles to form Pickering emulsions. This prevents reverse micelle formation entirely and has been shown to enhance the retention of the fluorophore resorufin [33].

However, since these alternative surfactants may reduce transfer mediated by micelle formation, they will not be able to inhibit the transfer of molecules that partition directly into the oil phase, such as alcohols. While it has been suggested that Pickering emulsion may form a barrier at the droplet interface to prevent partitioning, such emulsions are often not fully covered, leaving sufficient space for molecules to enter the oil phase [33,49]. Similarly, the effect observed with bovine serum albumin has only been shown to apply only to large molecules like fluorophores and cannot be translated to small molecules [17]. We therefore expect that the suggested remedies will not prevent the transfer of directly partitioning molecules. However, it remains to be shown if they can present a solution for molecules that do not partition directly, but transfer nonetheless. Future work systematically testing these strategies on different molecules could provide insight about their utility in preventing transfer.

Based on our work, we recommend that researchers carefully consider their molecules when designing droplet-based biological assays. Specifically, we encourage experimental validation of both compartmentalization and its biological effects, especially when working with small, amphiphilic molecules. When strict compartmentalization is required, primary substrates with low  $\log P$  values ( $<1.5$ ), such as sugars and glycerol, are preferable. If a substrate is shown to transfer, researchers should test if the molecules directly partitions into the oil. In such cases, a fully perfluorinated oil (e.g. FC-40) might reduce transfer sufficiently. If micelle shuttling is suspected, using transfer-resistant surfactants or nanoparticles can help eliminate transfer. For metabolic products prone to transfer, reducing the cell-to-droplet ratio can dilute transferred molecules, lowering the effect on other cell-containing droplets. While no single strategy is likely to fully eliminate molecule transfer, careful consideration of the experimental design may significantly reduce transfer and ensure robust experimental outcomes.

To our knowledge, this is the first study to demonstrate the biological effect of the transfer of common substrates and (by)products encountered in high-throughput screening experiments. The biosensor assay can be adapted to test other molecules, provided that a consuming strain is identified for the chosen molecule. While our approach offers a simple and accessible framework to assess the effect of molecule

transfer, it has limitations. The setup was only tested on a limited range of molecules, and future studies might include more nucleic acids, or other biologically relevant molecules. Additionally, while most studies use monodisperse droplets generated in microfluidic systems, we tested polydisperse emulsions generated by vortexing. The size variation in our setup might exacerbate the transfer by additional systemic effects, such as Ostwald ripening. We showed that droplet ageing cannot explain the difference in transfer between different molecules and ageing did not induce transfer of glucose (Supplementary section S4.2, Figure S4.2). This suggests a minor role of Ostwald ripening for transfer, but a more rigorous study of droplet ageing could elucidate the effects introduced by polydisperse emulsions. We therefore cannot readily extend the generated data to the more commonly used monodisperse emulsions. Lastly, since the scope of this study was the biological effect of molecule transfer, we did not do more rigorous testing of the mechanisms governing transfer. Such studies have been performed previously and directly led to the development of novel surfactants limiting transfer [14,20,33]. This study demonstrates that molecule transfer can significantly affect the experimental outcome of biological high-throughput experiments with potentially broad implications for mutant selection or growth assays using droplet systems. By giving practical recommendations for researchers, we encourage researchers working with droplet setups to carefully consider the potential of substrate or product transfer and their effects on neighbouring environments and to validate compartmentalization of the molecule in question.

## 4.4 Materials and Methods

### 4.4.1 Microbial strains and growth conditions

Table 4.2: Strains used in this study

| Strain                               | Description                         | Genotype                                                        | Reference |
|--------------------------------------|-------------------------------------|-----------------------------------------------------------------|-----------|
| <i>S. cerevisiae</i><br>CEN.PK113-7D | Common Laboratory strain (wildtype) | <i>MATa MAL2-8c SUC2</i>                                        | [28,50]   |
| <i>S. cerevisiae</i><br>CEN.PK113-5D | CEN.PK113-7D (uracil auxotroph)     | CEN.PK113-7D<br><i>ura3-52</i>                                  | [28]      |
| <i>E. coli</i> MG1655                | K-12 wildtype strain                | F <sup>-</sup> $\lambda^-$ <i>ilvG<sup>-</sup> rfb-50 rph-1</i> | [51]      |
| <i>L. cremoris</i><br>MG1363         | Common laboratory strain (wildtype) | -                                                               | [52,53]   |

*Saccharomyces cerevisiae* CEN.PK113-7D and CEN.PK113-5D were grown on high concentration synthetic medium (HCSM), containing per litre: 10 g  $\text{KH}_2\text{PO}_4$ , 5 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ , 6.6 g  $\text{K}_2\text{SO}_4$ , 9 g  $\text{CH}_4\text{N}_2\text{O}$  (urea), trace elements and vitamins [54].

When required, it was supplemented with  $150 \text{ mg} \cdot \text{L}^{-1}$  uracil [55]. *Escherichia coli* MG1655 was grown on M9 minimal medium, described by Maniatis *et al.* [56]. *Lactococcus cremoris* MG1363 was grown on CDMpc, described by Price *et al.* [57]. Glucose, ethanol, and acetic acid were used as carbon sources, in the concentrations as indicated. When required, single amino acids were left out of the final medium to test their transfer.

The *S. cerevisiae* strains were pre-cultured in HCSM + 2 wt% glucose at 30 °C in 100 mL round-bottom shake flasks in a shaking incubator. The *E. coli* MG1655 strain was precultured in M9 + 0.2 wt% glucose in aerated, 50 mL Greiner tubes at 37 °C in a shaking incubator. The *L. cremoris* MG1363 strain was pre-cultured in CDMpc + 0.09 wt% glucose in 50 mL Greiner tubes at 30 °C in a standing incubator. Freezer stocks were prepared by growing the strains in the indicated medium with appropriate carbon source and storing the stationary culture (exponentially growing for *S. cerevisiae*) at -80 °C with 20 vol% glycerol (*S. cerevisiae* with 30 vol%).

#### 4.4.2 Quantification of metabolite partitioning

To quantify the extent of metabolite partitioning, metabolites were diluted in 1x phosphate buffered saline (PBS) and their concentration was determined using high-performance liquid chromatography (HPLC). The concentration of each molecule was chosen according to the resolution of the HPLC. Hundred microliters of each metabolite solution was added to 900  $\mu\text{L}$  HFE7500 oil (3M, Two Harbors, MN, USA), FC40 oil (3M, Two Harbors, MN, USA) or mineral oil (Merck, Darmstadt, Germany) and vortexed (Vortex-Genie 2, Scientific Industries inc, Bohemia, NY, USA) on maximum speed for 5 min. Afterwards, the concentration of the molecule in the liquid phase was determined using HPLC. Statistical analysis was performed on the whole dataset, one-tailed t-tests, assuming not equal standard deviation among the samples. One-tailed was chosen to indicate that only the partitioning of the molecule from water phase to oil was tested.

#### 4.4.3 Generation and quantification of cell growth in microdroplets

Cell-containing emulsions were generated according to [6], with slight alterations. Cells were pre-cultured to stationary phase and washed once with 1x PBS. Afterwards, the cell concentration was determined using flow cytometry (Accuri C6, BD Biosciences, San Jose, CA, USA). Based on the measured concentration, the cells were diluted to a final concentration of  $3.0 \cdot 10^7$  cells/mL using the corresponding growth medium and carbon source. From this aqueous phase, 300  $\mu\text{L}$  was added to 700  $\mu\text{L}$  of either HFE7500 + 0.2 vol% PicoSurf 1 surfactant (Sphere Fluidics, Cambridge, UK) or FC40 oil + 0.4 vol% PicoSurf 1 surfactant. Afterwards, emulsions were generated by agitating for 5 min at 2500 rpm using a multi-tube vortexer (DVX-2500, VWR, West Chester, PA, USA) and placed on ice. The generated droplets had

a representative volume  $\pm$  SEM of  $37.6 \pm 6.9$  pL (HFE7500) and  $36.9 \pm 6.5$  pL (FC40) (Supplementary Section S4.1). With this droplet size and the above-mentioned cell concentration of  $3.0 \cdot 10^7$  cells/mL, there will be on average 1 cell per droplet.

The prepared emulsions were covered with parafilm to ensure sufficient oxygen transfer and incubated in a standing incubator at 30 °C (*S. cerevisiae* and *L. cremoris*) or 37 °C (*E. coli*) for 72 h (*S. cerevisiae* and *L. cremoris*) or 48 h (*E. coli*). After incubation, the emulsions were added to a 15 mL Greiner tube and broken by addition of 1 mL PBS and 1 mL perfluorooctanol (PFO, Alfa Aesar, Ward Hill, MA, USA) with gentle mixing. The resulting water layer was transferred to an Eppendorf tube, and the cell concentration was measured using flow cytometry.

#### 4.4.4 Visualization of pH transfer in microdroplets

To visualize pH in microdroplets using microscopy, a 5(6)-carboxyfluorescein (Merck, Darmstadt, Germany) solution was prepared by adding 4 mg 5(6)-carboxyfluorescein to 100  $\mu$ L of 100 % ethanol and then adding 900  $\mu$ L of demineralized water. This was added to 100 mL of M9 for a final concentration of 40 ppm carboxyfluorescein. Solutions of M9 with the indicated carbon source and pH (pH set with either 2.5 M HCl or 5 M NaOH) were used to generate emulsions as indicated above. The resulting emulsions were mixed, if required, and imaged immediately, as well as after incubation for 24 h at 30 °C in a standing incubator, using fluorescence microscopy (Zeiss Imager Z1, Zeiss, Jena, Germany) at 200x magnification. The fluorescence of individual droplets was analyzed using a custom Python script (supplementary section 4).

#### 4.4.5 Estimation of ethanol partitioning in emulsions

To assess the effect of surfactant concentration on ethanol partitioning, 300  $\mu$ L of aqueous phase containing PBS with 100 mM ethanol was added to 700  $\mu$ L of HFE7500 oil + PicoSurf 1 surfactant at the indicated concentration. Emulsions were generated as described above and incubated down at room temperature for 16 h to allow for settling of the microdroplets at the top. From the emulsions, 400  $\mu$ L of the oil was removed from the bottom and added to 100  $\mu$ L PBS without agitation. The solution was incubated at room temperature for 6 h, and the resulting ethanol concentration in the PBS was measured using HPLC.

## Acknowledgements

This work was funded by Delft University of Technology. T.d.K. was financed by the Netherlands Organisation for Scientific Research (NWO), as part of the research program ENW-M2 with project number 15507. We kindly thank Michelle Rossouw for stimulating discussions and for critical reading of the manuscript.

## Author contributions

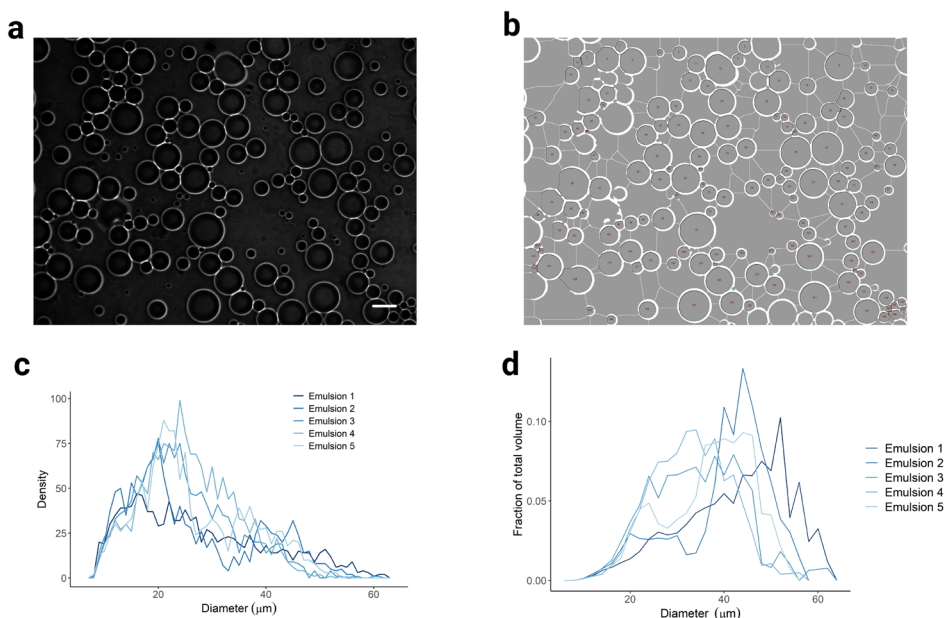
TF: Validation, Methodology, Formal analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization. TdK: Investigation, Review & Editing. RvT: Conceptualization, Supervision, Writing – Review & Editing.

## Supplementary Information

### Section S4.1: Droplet size estimation pipeline

To estimate the average size of generated microdroplets, we followed the protocol described in the Materials and Methods section. We imaged the resulting polydisperse microdroplets at 200x magnification using nine images per emulsion (Supplementary Figure S4.1a). These images were then processed with a custom imageJ pipeline, that applied a watershed algorithm to segment the droplets (Supplementary Figure S4.1b). Very small droplets ( $< 2 \mu\text{m}$ ) and droplets from the border of the picture were excluded from the analysis.

The diameters of the segmented droplets were extracted and pooled over all images. Since the vortex-based droplet generation method generates polydisperse emulsions, a wide range of droplet sizes was observed. Although smaller microdroplets ( $< 20 \mu\text{m}$ ) are more abundant (Supplementary Figure S4.1c), due to a low volume of these microdroplets, they contribute less to the total volume of the aqueous phase (Supplementary Figure S4.1d). In contrast, larger droplets account for a greater share of the volume. To calculate the representative droplet size, we analysed droplet size distribution normalized by the volume fraction. Specifically, for each emulsion, we identified the five droplet size bins contributing the most to the total volume and averaged these sizes. This value was taken as the volume-weighted average droplet size. We repeated this process across multiple emulsions to obtain an estimate of the variation in droplet size (Standard error of the mean, SEM). The resulting representative droplet size for the volume-weighted size distributions is  $37.6 \pm 6.9 \text{ pL}$  ( $40.6 \pm 2.7 \mu\text{m}$  diameter).



**Supplementary Figure S4.1: Droplet size analysis pipeline.** a) Microscope image of microdroplets. Scale bar: 50  $\mu\text{m}$ . b) Droplets segmented using watershed. c) Histogram of droplet sizes in five emulsions. d) Diameter distribution normalized by total volume. The top 5 most occurring diameter bins of each emulsion are averaged to calculate the representative droplet size.

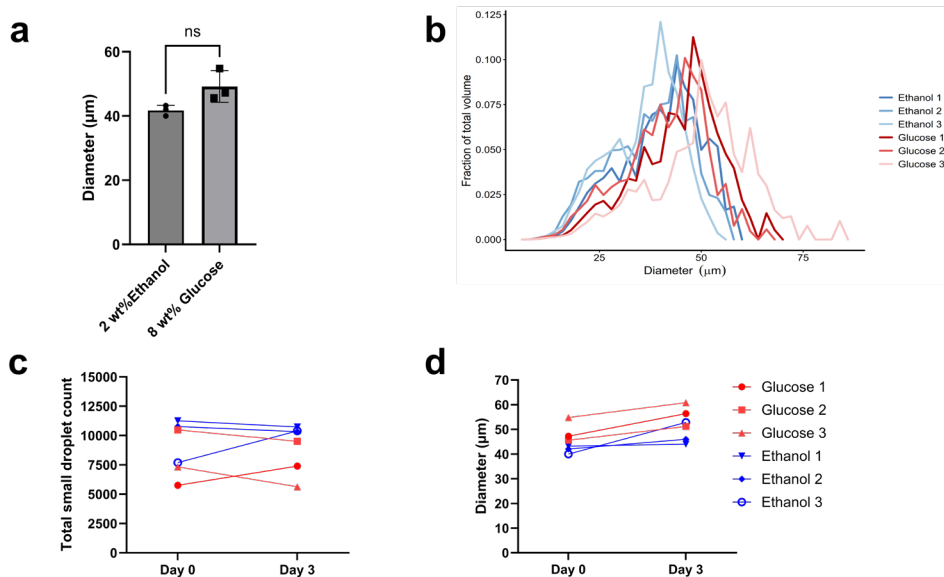
## Section S4.2: Differences in droplet generation between substrates

Our system uses bulk emulsions that display a wider size distribution than monodisperse emulsions generated with microfluidic devices. To test if the composition of the aqueous phase influences droplet size or ageing for this method, we prepared emulsions containing either 2 wt% ethanol or 8 wt% glucose.

We analyzed the distribution of droplet sizes using the ImageJ particle size estimation pipeline described in Supplementary section S4.1. Both the average droplet size (Supplementary Figure S4.2a) and the size distribution (Supplementary Figure S4.2b) were similar between glucose and ethanol containing microdroplets, indicating that the selective transfer ethanol cannot be explained by differences in microdroplet generation.

Next, we explored whether ethanol might influence droplet ageing over time, explaining its selective transfer. Specifically, we were interested if the average droplet size would change with either molecule, or if the number of small droplets, potentially acting as molecular shuttles, would change between the different

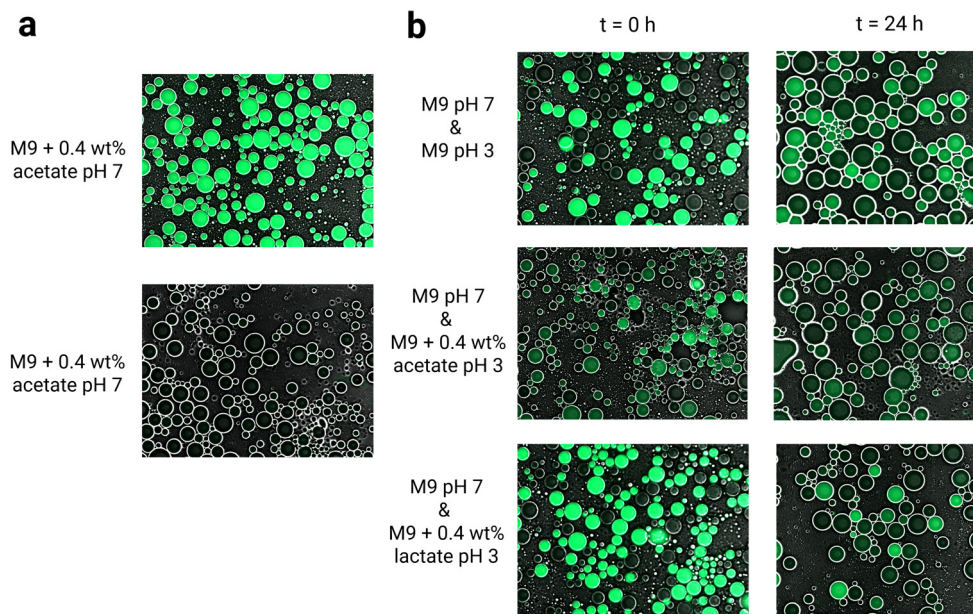
emulsions over time. Neither the number of small droplets (Supplementary Figure S4.2c), nor the size (Supplementary Figure S4.2d) showed a difference between both different emulsions that could explain the selective transfer of ethanol, suggesting that the transfer of ethanol is more likely related to its molecular properties rather than a property of the emulsion system.



**Supplementary Figure S4.2 : Size and ageing behaviour of emulsions containing ethanol or glucose.** a) Average size of microdroplets. Microscope images are taken of each emulsion and the imaged droplets are measured for size as a fraction of total volume. Three such emulsions are prepared and characterized (bars: average  $\pm$  SD). Statistical test: student t.test, unpaired, two-tailed,  $\alpha=0.05$  ns:  $p=0.067$ . b) Resulting distribution of all assessed droplets within each emulsion as a fraction of total volume. c) Amount of small droplets ( $< 2 \mu\text{m}$ ) per picture upon generation and after three days. The number was assessed with image analysis from microscope images. d) Average droplet size upon emulsion generation and after 3 days.

## Section S4.3: Imaging stained droplets

To support the histograms shown in figure 5, we include representative fluorescence microscopy images of the same droplet emulsions used to generate the histograms (Supplementary Figure S4.3).



**Supplementary Figure S4.3: Representative fluorescence microscopy images corresponding to the droplet fluorescence histograms shown in Figure 4.5.** Droplets contained medium and the pH-dependent fluorophore 5,6-Carboxyfluorescein that shows fluorescence at pH >5 were generated and imaged. a) Representative images of single emulsions of M9 at either pH 7 or pH 3. b) mixtures of emulsions containing M9 pH7 + M9 pH3 and, if applicable, a weak acid. Representative images at t = 0 h and t = 24h.

## Section S4.4: Segmentation of stained droplet populations

To quantify the droplet fluorescence, we used an image analysis pipeline, based on droplet segmentation. We obtained images by fluorescence microscopy, labelled them using the watershed algorithm on the fluorescence channel and then calculated the mean fluorescence for all droplets in each image. For each condition, we analysed >250 droplets. The segmentation was manually reviewed and corrected using Napari [58].

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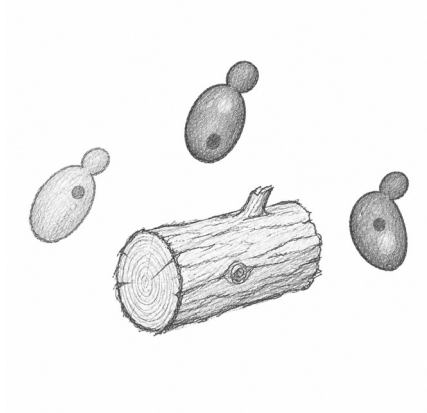
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Do not all charms fly

At the mere touch of cold philosophy?

John Keats, 1819



# **Chapter 5**

## **Outlook and Valorization aspects**

Over the past decade, defined synthetic co-cultures have emerged from academic concepts to potential alternatives to monocultures in industrial biotechnology. They offer potential solutions to the most pressing challenges of current bioprocesses, including high metabolic burden through pathway imbalances, enzyme limitations of chassis organisms, and the utilization of complex feedstocks, as discussed in **Chapter 1** [1]. This development is timely, since the nascent bioeconomy is more than ever expected to offer viable solutions to replace fossil-based processes, but for many potential productions faces challenges in reaching economically required product titers, productivities and yields [2].

One particular area in which co-cultures have potential to outperform traditional approaches is the conversion of biomass into chemicals and fuels [3]. For example, bioethanol, the largest-volume product of industrial biotechnology, is almost exclusively produced from corn starch and cane sugar. Further expansion of this process meets limitations related to land use for other human needs [4]. This limitation could be avoided by utilizing residues, such as lignocellulosic biomass [5,6]. However, utilizing complex feedstocks like lignocellulosic biomass is technically and economically challenging due to the current multi-step chemical/physical, enzymatic and microbial conversion processes of this complex material [7]. Unlocking this resource has tremendous potential, which has prompted much attention to novel strains or pretreatment methods [6].

In laboratory studies, use of defined co-cultures of microorganisms has been shown to improve certain shortcomings that monocultures or generalist strains face – for example by consuming all lignocellulosic components in parallel instead of sequentially [8]. Mixtures of specialist strains, each able to convert only a single sugar, also show better functional stability during prolonged cultivation than generalists [8,9]. This presents key industrial advantages, since it enables recycling of biomass between cycles, which can increase volumetric productivity in large-scale processes [10]. However, there is a stark contrast between increasing academic interest in co-cultures for conversion of lignocellulosic hydrolysates and the current application of such processes at commercial scale [6]. This contrast highlights unresolved ecological and engineering constraints that must be addressed before co-culture-based lignocellulose conversion can be deployed at scale.

The narrative around specialist co-cultures has mostly advanced through case-specific reports, since the conditions at which a co-culture of specialists can potentially outcompete a generalist monoculture have not been rigorously defined yet [8,11]. Therefore, there is a need for a theoretical framework that establishes criteria for the success of co-cultures over monocultures, similar to those developed for co-cultures with assimilatory product pathways [12]. A set of rigorously formalized criteria that define contexts in which co-cultures are an economically valuable alternative to

current monoculture-based processes would be highly valuable to guide academic and industrial research. These criteria would be based on quantitative parameters that describe growth kinetics, growth energetics, and resource allocation in individual strains, as well as parameters that describe interactions between strains. In application-inspired research, combinations of strains in defined co-cultures are generally not based on naturally occurring consortia, allowing unexpected interactions to appear during the process. These interactions must be characterized by their effect on co-culture performance and, if negative, mitigated. One such interaction was hypothesized to govern a tripartite co-culture of a glucose-, xylose-, and arabinose specialist, which led to growth stalling of the xylose specialist and growth restoration after evolutionary engineering [8]. Elucidating the molecular basis for this interaction that resulted in the stalling growth was a major part of my four-year research project. Unfortunately, despite genome sequencing and extensive reverse engineering efforts, a full characterization of the effect has remained elusive. A key factor confounding the research was an unexpected and substantial impact of the composition of the sugar mixture on fermentation kinetics. This prevented the isolation of the effect of the co-culture context within the available time. However, having identified this context dependency, it does provide an interesting avenue for future efforts on this scientifically challenging and industrially relevant subject. Progress in this regard could enable the engineering of more robust co-cultures and pave the way for eventual large-scale implementation [6,13].

Differences in the substrate-conversion kinetics can present challenges for the design of efficient co-cultures. For example, the constituent strains often present different specific growth rates, as a consequence of which overall volumetric productivity is limited by the slowest-growing strain [14]. To mitigate this challenge, **Chapter 2** explored selective sedimentation of the slowest-growing strain in a binary sequential-batch co-culture of a glucose-specialist and an arabinose-specialist yeast strain. Increased biomass retention of the slow-growing arabinose-specialist strain across sequential-batch cycles enabled fully parallel consumption of the two sugars and strongly reduced the time required for complete substrate utilization. While this approach illustrates a potential application of co-cultures in mixed-substrate environments, the high broth viscosity encountered in these settings would translate into additional challenges for this strategy [10]. Future research should therefore focus on accelerating sedimentation, for example by engineering larger and more rapidly sedimenting aggregates of the arabinose-consuming strain by adaptive evolutionary engineering [15]. Results could potentially be extended to a three-member consortium consuming all three major lignocellulosic sugars. Advances in this area could enable fast, parallel consumption of complex feedstocks through selective sedimentation by co-cultures in sequential-batch systems [7].

If fully parallel consumption of available sugars is the target of the co-culture, negative ecological interactions should be avoided. However, in practice, the success of the co-culture may depend on positive interactions that must be preserved. For example, a cross-feeding, mutualistic interaction promotes the occurrence of non-contributing cheaters, which can disturb the interaction between the cooperating strains and reduce overall culture growth [16]. **Chapter 3** explored this phenomenon and defined a single parameter, the time of first contact between cooperators as the determinant for co-culture success in spatially defined systems. While tested on agar plates, the results provide a simple framework for cooperative networks in similar systems, such as biofilms [17]. The structured organization and toxin tolerance of biofilms have led to their increasing use in bioreactors, where they may enable higher productivities and the co-cultivation of strains with distinct nutrient requirements [18,19]. Therefore, a better understanding of the determinants of ecological success in spatially defined communities may have indirect implications for their application. Future research should extend the findings to other systems like biofilms and explore strategies for how they can be used to limit cheater proliferation in such systems.

Since the ecological interactions governing even simple consortia remain poorly understood, an alternative strategy to design functional co-cultures may be high-throughput screening to identify candidates. Encapsulating single cells or consortia in microdroplets can enable the cost-effective screening of up to  $10^{10}$  candidates per mL volume, which represents a significant gain in throughput over more manual systems, such as microtiter plates [20,21]. However, the approach critically relies on effective compartmentalization of each microdroplet, such that substrates or metabolites cannot transfer between neighboring droplets. **Chapter 4** demonstrated that some common microbial metabolites, including ethanol, can transfer between droplets and thereby compromise the compartmentalization of the system and altering local carbon availability. While transfer was demonstrated and characterized for several common biological molecules, mitigation strategies remain unexplored. Future research should therefore focus on developing surfactants that limit transfer and identifying parameters that allow for better a priori estimation of transfer risk. In addition, the transfer effects should be systematically evaluated in monodisperse droplets, which are widely used in microfluidic research. Establishing clear guidelines for transfer risk and prevention could enable improved high-throughput screening methods both for single cell libraries, as well as co-culture combinations.

For most envisioned applications, defined co-cultures have not yet reached maturity. The primary challenge lies in the added ecological interactions that accompany a multi-strain system, which require maintaining an optimal ratio of co-culture members throughout the process. These interactions can affect the process outcome, a problem which is exacerbated in processes that require strains to cooperate. To better understand emergent ecological dynamics, the field would benefit from

improved monitoring methods that allow for real-time tracking of co-culture ratios [22]. If co-culture dynamics can be tracked in real time, maintaining optimal ratios could be achieved by exerting control via established process control methods [23]. Real-time tracking coupled with machine learning algorithms have shown promise to optimize co-culture ratios for maximum product output [24]. Targeting more application-oriented challenges, such as added complexity in the seed-train, will help to define the economic viability of this technology more rigorously and make co-cultures increasingly accessible for large-scale testing. Stable, modular, and robust co-cultures could unlock a plethora of now economically infeasible biomolecules and have the potential to expand the substrate range to second-generation feedstocks for large volume products.

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# Acknowledgements

It takes a village to raise a PhD.

Many people, including me, underestimate how different the PhD venture is from any other career choice. Most of us go into this because it sounds like a fun adventure, and a chance to play the real game. Yet, it is four years of your life in which you continue to go to university, while many of your friends already start earning high salaries, forming families, and pursuing what they have imagined for their early thirties. It generates a different kind of pressure, a lonely one, which is how a PhD can feel sometimes. This may begin to explain the mental health crisis striking PhD students across scientific fields, which is exacerbated by personal life continuing around you. The only remedy is community, something which I have been blessed with throughout my time here, and to whom I dedicate this section of my thesis. It is people around me that have shaped this thesis, by sharing intensely good times, picking me up during struggle and helping me get to the finish line. I would like to express my gratitude to the people in the following sections, and to everyone who I have met that shaped me as a person during the past four years.

First and foremost, I want to express how blessed and grateful I am to be a healthy, independent adult, capable of critical thought and risk-hungry enough to consider pursuing a PhD. I am too agnostic to speculate who dealt me these cards, but I want to express my gratitude to good fortune and the environment that fostered this.

I have learned that the success of a PhD hinges on the candidate and the supervisors being fully aligned with the mission and fostering an atmosphere in which personal and professional things can be shared and addressed immediately. I have been blessed with my two supervisors *Rinke van Tatenhove-Pel* and *Jack Pronk*, who are both passionate about personal development and are both a good mix of detail-oriented and pragmatic. It would be an understatement to say that they are great researchers and have been both inspiring and guiding in my scientific development. I would like to thank Rinke for her constant support and always putting her PhD students as the highest priority, even when she has a full calendar. It was immensely helpful how fast she sent back drafts, something that every PhD candidate wishes for when time is running short. She also gave good emotional support, and although I can be stubborn at times, always took the time to discuss things out. My gratitude

extends to my promoter, Jack, who supplied not only very detail-oriented feedback to my drafts, but also much-needed enthusiasm and novel ideas for the project. Notwithstanding his busy schedule, Jack took a full supervisor role when Rinke was on maternity leave, which I am very grateful for. It is evident that both supervisors have a clear priority on mental health and show compassion and flexibility when private life takes a toll on work well-being. I am grateful that they could spot some early signs of overwork in me and interfere immediately. They both give priority on the candidate finishing in four years, which of course is department policy, but it was always reassuring that my supervisors were deeply convinced of it. Thank you!

No other person apart from my supervisors has been so instrumental in me finishing this thesis than my dear colleague and friend *Michelle Rossouw*. Michelle has been a great support inside the lab, in which together we championed overnight sampling schedules, keeping each other awake by playing cards, and her other countless contributions to almost every chapter of the thesis. She was always the first person to get enthusiastic about new results, and a perfect conversation partner to discuss seemingly contradicting data. Her passion for rugby and her home country South Africa always cheered me up and I wish her and her partner Heinrich all the best in the coming years. Dankie vir jou ondersteuning!

I would like to thank *Sagarika Govindaraju*, who is a dear colleague and friend of mine and has supported me from the moment I arrived at the university. Back then, IMB was smaller and we, as Rinke's first PhD students, were working closely together, culminating in a week-long course in Amsterdam, which was hilariously off-topic for us, but resulted in a friendship that lasts till today. I am incredibly happy that I was paired with her to write the review, since we had lots of fun doing it, especially during the final push after two years. All the best with your postdoc!

I am grateful to *Thomas Visser* and *Tom de Kanter*, also PhD candidates of Rinke and, in Thomas' case, my office mate. Thomas and Tom both are authentic in a way that is contagious. Thomas has a very compassionate side and is possibly the best person to turn to to vent or to be distracted with a great story over coffee. Tom is creative in a way that may be quite unusual to find in a technical university and only speaks to his passion for new experiences and drive to give everything a personal touch. Both have been a big inspiration for me, and I am blessed to count them as friends and co-workers.

Further, I want to express my gratitude to the sometimes intensive but always fun discussions during the 'Defined Consortia (DECO)' meetings. Specifically, I want to thank *Djordje Bajić*, *Minke Gabriëls*, and *Uros Gojkovic* for the good energy and fresh perspectives that they brought to these meetings and wish them all the best for the future.

In 2022, IMB was much less crowded, and so I want to thank the people who have

been there at the beginning. My immense gratitude goes out to *Denzel Ignacia*. Who can say they worked side-by-side with their best friend in the most intensive period of their lives so far? I can, and it was an incredible pleasure to experience such a level of mutual support. Denzel has shaped me as a person in a way not many others have, and I truly missed him in my last year, when he was occupied with something even more important. He is a continuous inspiration as scientist, musician and friend. *Marcel Vieira Lara* and *Rozanne Stroek* were also instrumental in shaping my early experience at the institute, and I am very grateful for their support and their continuing friendship. Marcel is incredibly multifaceted, both as a researcher, and in how he expresses himself in his private life and has been a great inspiration for me. He dares to challenge narratives in a very refreshing way, and has an anecdote, advice or a joke on almost every topic. The Leuven conference trip will always be a hilarious memory for me. Rozanne is very compassionate, caring, and creative and has become a great friend of mine, even when she does not work at the institute anymore. Her moral convictions and passion for music are a continuous inspiration. Thank you both for the amazing time we spent together!

Next, I would like to thank my Paranympths, *Gabriela van Leersum* and *Malte Marquardt*. They are both very authentic in their own way and it fills me with immense gratitude to be their friend and co-worker. Gabriela is an organizing talent and has many interests, but somehow always finds time for a coffee or a longer chat, a privilege which I shamelessly often used. Malte and I, besides our German nationality, share a highly diverse set of convictions, interests and weird cultural references. Both have incredible humor, and a way to be silly in one moment and empathetic in the next, which is only rarely encountered. Thank you both for your support and for agreeing to take office as Paranympths!

It is an open secret that the engine of PhD students at this institute would grind to a halt without excellent technical (and emotional) support from the staff members. First, I would like to thank the MSD team with *Apilena Sapioper*, *Jannie Kempff-Gruwel*, and *Gea du Chatenier-de Bruijn*. Not only are they fantastic at what they do, they also go out of their way to help, by catering to the chaotic needs of stressed-out PhD students and by always offering a listening ear. Thank you for being there for us and being yourself, it was a blessing to work with you. I would like to extend my gratitude to *Nayyar Kalpoe*, who has always been supportive in the lab, and has often improved my mood with a good story or a joke.

Inside IMB, I would like to extend my gratitude to the technical and educational staff consisting of *Erik de Hulster*, *Clara Carqueija Cardoso*, *Susan Bastiaans*, *Marijke Luttik*, *Ilja Westerlaken*, *Christiaan Mooiman*, *Marcel van den Broek*, *Marieken Tack*, and *Flip de Groot*. Erik, as the linchpin of the fermentation lab, is the person you wish to be there when a bioreactor run is about to fail, or to invent a MacGyver-style solution for any problem you can encounter. But he is also a great

support, and an excellent conversation partner, not only in the lab but also when wading through the Waddenzee, and I am grateful for having worked with him. Clara, being responsible for the finances and the molecular biology work, is super reliable, approachable, and generally a person you can trust with any question. I am very grateful to have shared many coffees, discussions and laughs with her. Susan, as the secretary of the section, has never failed to cheer me up with a goofy story, a pointy joke or some good-hearted advice and I truly wish her all the best in the coming period. Marijke, next to her excellent technical understanding of all protocols in this lab, has an incredible talent for listening. When sharing something with her, it always felt that she created a safe, non-judgmental environment and was truly concerned about showing support. Finding such a talent in a technical university is not a given, and I am very grateful for the many conversations we had. While Ilja and I have not shared a lot of work together, I got to know her intensely over many coffee breaks, in which she always inspired me with her non-serious way of looking at life, her contagious laugh, our shared love for Panama, and her on-point social commentary. I am grateful for the unique perspective that she gave me. Christiaan, as the other fermentation technician, is just as reliable as his peer, which has saved me several times, even at inconvenient hours, for which I am very grateful. Marcel and I share a love for sports competitions, hilarious stories, and bioinformatics tools and has been my first person to approach for advice in either of these departments. He has inconsumable patience with explaining the workings of his data analysis tools, and is a very reliable colleague and friend, for which I am very grateful. Marieken supervised the BBT1 course, for which I TA'd, and I consider her an incredible teacher, colleague and friend. I really cherish the good memories I have with her. Lastly, I am grateful for working with Flip, who, apart from being an excellent technician, never disappoints with an anecdote or comment on almost any topic during lunch. Thank you all for your immense support and for being authentic, approachable and fun to be around with, the department is very lucky to have you. I will miss working with such a competent team.

Within IMB, there were many of my PhD and Postdoc colleagues that I had the pleasure of meeting and being inspired by. While everyone was of course busy with their personal project, it still felt like we were crew on the same ship. *Effie Leijten* is a person who has seen me struggle and managed to pick me up with good-hearted support and a story over a cup of coffee. She is a very open and empathetic person with a contagious laugh, that I have shared many good memories with and whose friendship fills me with gratitude. *Marieke Warmerdam* and I had the joy of organizing the lab retreat together, which was an astounding success and where I got to know her as a creative, fun-loving organizing talent, which adds to her being an inspiring scientist for me. *Nicole Bennis* and I shared an office in the beginning of my PhD, and she is a warm-hearted, pragmatic person, that loves the small joys in life, such as a 'delicious golden liquid on a terrace'. *Rosalie Wouters*, owing to her

humility and great, often self-deprecating, humor has often cheered me up when experiments went south, for which I am grateful for. *Miriam Cammaert* has a talent for laughing about little things and is a great presence during lunch and in the lab, which I have always appreciated. I am also very grateful for having *Marc Pedersen* as an office mate, who never disappointed with a fun anecdote from Denmark and who has an incredible love for bioreactors. He is a very patient teacher and a fantastic engineer, who has taught me a lot. *Marcus Young* and I knew each other before, and it is admirable how he managed to integrate himself so fast within the IMB community. I am very grateful for his authentic humor, positive outlook, and his wealth of funny stories from his past careers. Lastly, I want to express gratitude for *Marta Cucchi*, who possibly has the driest humor you can think of, and can spark a discussion about any topic at the lunch table, although she has been here only a short while. For all of you I am grateful for having shared laughs, coffee, walks, intense lunch discussions, emotional lab days and for shaping me in one way or another.

People who know me well know that I also love to reach out to connect with other groups. The crew from BPE has become very dear to me. *Josh Flack* and I share a love for kitesurfing, DJing and endless discussions about math heavyweights. While I cannot quite reach his level of extreme sporting, I am grateful for the good memories with him, and for all the times he came peeking into my office to talk kitesurfing or events in the biotech sector. I am also grateful for the positive vibes that *Joana Carlos Pimenta* has brought to my days, with her hilarious, often self-deprecating, humor and her outlook on life. Positive vibes also always came from *Marika Zegers* and *Meryl Copray*, who are very guilty of making me stay too long in the coffee break, since our conversations often spanned multiple topics at once, including current gossip at the institute. I am grateful to *Héctor Maldonado de León*, as I know him as a very resilient, hard worker, who always remains humble. His love for his home country, biking, BBQing and his drive have been an inspiration for me from the beginning on. The entrepreneur course without Hector and Marika would have been pointless. Beyond BPE, I want to express my gratitude to *Natalia Nadal Alemany*, who has become a dear friend of mine, after spending 2 years teaching the practicals together. She is an incredibly caring, funny, and smart person, who has shaped me in many ways, including the inspiration to start with Bachata dancing. Further, I would like to thank *Rachel Los* and *Timon Idema* for the fun collaboration on one of the projects of this thesis. Especially Rachel has been a great colleague and inspiring in her drive and moral stances. I also want to thank *Miriam Prior* for her empathetic discussions, our common distrust of anything flashy in Biotech, especially the 'dire wolfs', and generally for being a smart, bouncy person. Her incredible breadth of knowledge in philosophy and ethics makes her a fantastic discussion partner and I am grateful for the memories of our nights exploring the music scene in Den Haag. Lastly, I want to thank *Charles Moritz*, for being instrumental in shaping my experience in Den Haag, his passion for surfing and science, and for being a good

friend. He supported me in crucial moments, and I cherish many good memories of adventures in the Netherlands, California, Austria, and Croatia with him. All of you have a share in bringing this project to the finish line and were inspiring to me in many ways and I thank you for that.

I had the pleasure of teaching the Bachelor students *Stefan Vreeke*, *Floor Bleekveld*, *Sanne Diepen*, and *Fons Sluijter*. Often, they taught me things as much as I taught them, and I am very grateful for the contribution to the project they made. Apart from that, I applaud their drive and curiosity and wish them all the best for their way forward.

A somewhat unusual acknowledgement goes to the 'Get in the sea society'. They are an incredible and diverse group of people that I go swimming in the North Sea with every Saturday, throughout the whole year. They have provided structure in my week and are probably responsible for me not getting severely sick throughout the whole PhD. Particularly, I want to thank *Coen Jonkman*, *Milan Nossin*, *Katie Davis*, *Laoise Brennan*, *Alice Pavin*, *Will Murray*, *Anna Jensma*, *Ruud van der Kant*, *Rahya Moradi*, and *Rom van Stolk*. With many of them, I share more than the weekly swim, and I want to express my gratitude to them for being authentic, funny, empathetic, and instrumental for shaping my life in Den Haag. Thank you!

Similarly, I want to express my gratitude to the people I worked with at the non-profit organization 'Science meets Business'. This includes *Fleur van Eden*, *Willemijn Heinen*, *Max Green*, *Bastiaan Kluft*, *Vincent van der Wal*, *Frederique de Paus*, *Salomi Smyri*, and *Heba Klij*. Their entrepreneurial spirit is palpable and organizing these events with such a high-energy team was always a highlight of my month. Together, we not only brought together students and entrepreneurs from around the Randstad but also got the chance to see crazy event ideas come to fruition. I am grateful to have spent so much time with these people and wish them all the best in their endeavors.

The people I met during my time in the Netherlands that have now become good friends of mine I also want to express gratitude to. This includes *Eirin Sundby*, *Fabian Mulder*, *Xaviera Cárdenas*, *Subin Kim*, *Sebastian Rodriguez*, *Juliana Rincón*, *Francisca Mingram*, *Santiago Mingram Visser*, *Hariwa Wafai*, *Bryan Dowling*, *Bryan Blair*, *Merlijn Hulsenboom*, *Letizia Artioli*, *Constanza Ivaria*, *Rafael Rinaldo-Ivaria*, and *Clement Rinaldo*. I also want to thank my other friends from my home country, for providing me with a lifeline to Germany and for the countless visits. I want to express gratitude to *Lukas Kiefer*, *Lena Kiefer*, *Lisanne Hameleers*, *Florian Drüke*, *Philipp Ruppert*, *Max Kroppen*, *Anna Kreuz*, *Robert Schnüll*, *Robert Klöckers*, *Meike Münster*, and *Nick Machnik*. They have all contributed in their individual ways with support, inspiration or diversion. Without their ongoing support and presence, my life would not be half as interesting and fun, thanks a lot to all of you!

To all other people I met during my time here at this institute, who have in their own way impacted this journey, a heartfelt thank you. A special mention goes out to *Jenny and Mauricio* from the fellowship restaurant 'Sapori di Casa'. They are fantastic chefs, and always manage to elevate my mood with great, nutritious food and lots of laughs. Thank you!

Lastly, I want to thank my family, firstly *Birgit, Thomas, and Stephanie Fecker*. Es gibt nicht viele Moment im Leben, wo man in sich geht und einfach fühlt, wie dankbar man dafür ist, dass die wichtigen Menschen im Leben um einen herum existieren, so wie sie sind. Ich bin unglaublich dankbar, dass ihr mich bei bisher allen meinen Ideen nicht nur unterstützt habt, sondern mich angefeuert habt. Mama und Papa, ihr seid inspirierend, wie ihr Probleme angeht, wie ihr euch ausdrückt, wie ihr mir Zuneigung zeigt und mich anspornt, mein Bestes zu geben. Ich kann auf euch zählen, da ihr mir Freiraum gebt, mich auszuprobieren und in anderen Ländern mein Glück zu versuchen. Zuhause empfangt ihr mich immer mit Freuden aber auch so, dass es sich anfühlt, als wäre ich nie weg gewesen. Danke dass ihr mir Wurzeln gebt und mich in einer Weise aufgezogen habt, die mich immer inspiriert, Risiken einzugehen, wissend dass ich weich fallen werde. Ich bin sehr glücklich darüber, dass mich mit euch beiden zusammen und individuell so viel verbindet, besonders in der Musik und im Sinn für Kultur, und hoffe, das wird noch lange so bleiben. Steffi, du bist mir einer der wichtigsten Menschen in meinem Leben, was du natürlich weißt. Deine Authentizität und deine Art, wie du dein Leben angeht und immer deine Liebsten priorisierst, inspiriert mich. Ich hoffe, wir können immer so offen miteinander sprechen, wie wir es jetzt tun. Zusammen bin ich wirklich froh, auf so eine tolle Familie zählen zu können und ihr habt sicher einen nicht unbedeutenden Beitrag zu dem Erfolg dieser Arbeit beigetragen. Vielen Dank! Mein Dank geht auch an *Susanne Brüner*, die mich immer mit aufbauenden Worten und einer besonderen, spirituellen Perspektive unterstützt hat. Einen nicht zu unterschätzenden Beitrag zu meinem Werdegang als Wissenschaftler hatten auch *Karin, Rainer* und *Carsten Malisch*, ohne die ich wahrscheinlich überhaupt erst gar nicht diese Karriere eingeschlagen hätte. Ihr seid alle fantastische und inspirierende Menschen, für die ich sehr dankbar bin.

How glorious these sunbeams are !  
They seem to call Earth to the heavens

*Alexander von Humboldt, last words*

# Curriculum Vitae

Tobias Fecker was born on the 29th of June 1993 in Düsseldorf, Germany. After graduating from high school in 2012, Tobias went to pursue a BSc in Biological Sciences in Münster, Germany, graduating with a thesis awarded highest distinction in the department of nanobiotechnology with Prof. Francisco Goycoolea. In 2016, he went abroad for two years to work as a guest researcher in Santiago de Chile, focusing on protein-ligand interactions and molecular dynamics, together with Dr. César Ramirez-Sarmiento.

Tobias moved to the Netherlands in 2018, enrolling in the MSc. Program Molecular Genetics and Biotechnology at Leiden University. The master program entailed two research internships, one in the laboratory of Prof. Gilles van Wezel at Leiden University, developing a device enabling co-cultures of mammalian and bacterial cells and the other one as a joint internship at the groups of Prof. Jean-Marc Daran and Prof. Adrie Straathof at TU Delft, working on large-scale biotechnological process design of 2-phenylethanol. Tobias graduated in 2020 as the best graduate of that year. His work on 2-phenylethanol was awarded with the 'Krijn Rietveld memorial innovation award', given yearly by DSM-Firmenich and Leiden University to reward researchers who make extraordinary contributions in the fields of biotechnology and data science. As the team manager of the iGEM Leiden team, he participated in the largest worldwide competition for synthetic biology, and he and his team were awarded the trophy for Best Manufacturing project.

After graduating, he worked as process engineer at the Vaccine CDMO 'Batavia Biosciences', before joining TU Delft as a PhD researcher in 2022 under the supervision of Rinke van Tatenhove-Pel. From 2021 to 2025, Tobias was a volunteer at the non-profit organization 'Science meets Business Leiden', which aimed to support young entrepreneurs across the Randstad and inspire students to become founders.

Tobias is currently working as an investment analyst at World Fund, the largest climate-focused venture capital fund in Europe.

# List of Publications

- [1] H. Kleine-Brueggeney, G. K. Zorzi, T. Fecker, N. E. El Gueddari, B. M. Moerschbacher, and F. M. Goycoolea, "A rational approach towards the design of chitosan-based nanoparticles obtained by ionotropic gelation," *Colloids Surfaces B Biointerfaces*, vol. 135, pp. 99–108, 2015. doi: 10.1016/j.colsurfb.2015.07.016
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- [3] R. Los, T. Fecker, P. A. M. van Touw, R. J. van Tatenhove-Pel, and T. Idema, "Time of first contact determines cooperator success in a three-member microbial consortium," *ISME Commun.*, vol. 5, no. 1, pp. 1–10, 2025. doi: 10.1093/ismeco/ycaf004
- [4] T. Janković, T. Fecker, J. Daran, A. J. J. Straathof, and A. A. Kiss, "Advanced process design for large-scale de- novo 2-phenylethanol production via fermentation," *J. Chem. Technol. Biotechnol.*, vol. 100, no. 11, pp. 2271–2280, 2025, doi: 10.1002/jctb.70057.
- [5] S. B. Govindaraju\*, T. Fecker\*, M. Rossouw, J. T. Pronk, and R. J. Van Tatenhove-pel, "Unlocking the potential of defined co-cultures for industrial biotechnology : Opportunities and challenges," *N. Biotechnol.*, vol. 91, no. November 2025, pp. 89–99, 2026, doi: 10.1016/j.nbt.2025.11.009.
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\* These authors contributed equally

# Final Words

Throughout these four years, I have gathered much knowledge about microbes, their ecology and their application in biotechnology. But I could never shake the feeling that something was amiss from my understanding. Common scientific practice is of removing organisms from their natural environment, subjecting them to an artificial environment, for example a bioreactor, and then testing hypotheses on them. This reductionist method has certainly led to scientific breakthroughs.

But can this approach infer something about nature, which is defined by interaction rather than individuality? Is this different from calling a painting of a pipe, a pipe? Will this approach work with emergent phenomena? Can we knowledge turn into wisdom?

As scientists, our greatest asset is our curiosity, which I myself have taken much inspiration from throughout my career. For many scientists, myself included, this curiosity evolved when spending time in nature as a child. But somewhere along the way, I traded the curiosity-inducing experience of being in nature with a desire to describe it, by means of the so-called scientific method. Now, instead of seeing myself as part of the nature I am describing, I became an outside observer. Knowing more about its parts did not increase my understanding of the whole.

However, I realise, I am not alone. Many great minds have lamented this loss of the living band, as evidenced by the poems in this book. When reading how artists perceive nature, I begin to understand the missing ingredient in my quest for wisdom. My experience as an observer in the lab can be complemented by my experience as a participant in nature and vice versa.

Next time in a forest, I will think of yeast cells on trees and their molecular activity and how all of it contributes to my emotional experience in this forest. After all, knowing about cells does not have to diminish my awe in how it is all connected.



Apollo unveiling the statue of Isis.  
Engraving by Bertel Thorvaldsen for the  
dedication page to Goethe ("An Göthe.")  
in Alexander von Humboldt and Aimé  
Bonpland's *Ideen zu einer Geographie der  
Pflanzen* (1807)

Cover design: The front cover features Max Liebermann's painting *Lumberjacks in the forest* (1898) and depicts three lumberjacks working together in the forest, valorizing wood. The back cover presents a reinterpretation of the scene in which the three lumberjacks were replaced by a single, solitary figure, struggling to carry out work alone. Painting in public domain. Reinterpretation was generated by ChatGPT.

