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Design and engineering of artificial microbial consortia for biohydrogen production

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In natural microbial ecosystems the metabolic diversity of the organisms enables interaction among the community members and allows them to engage in syntrophic interactions. With regard to biotechnology, artificial microbial consortium engineering is used to improve productivities and yields of bioprocesses. However, to achieve supreme productivity or efficiency at industrial scale, defined ecosystems must be physiologically well-selected to meet eco-biotechnological demands. Here, we present an artificial microbial consortia design and engineering pipeline for developing dark fermentative biohydrogen production processes. The proposed pipeline might be considered as a blue-print for enhancing other bioprocesses that fundamentally face metabolic restrictions or kinetic limitations.

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Introduction

Microorganisms contributing with different functional roles to biogeochemical cycles are vital for sustaining Earth's biosphere. In nature, microbes exist in dynamically changing communities where they eco-physiologically engage in syntrophic interactions, forming functional ecosystems. However, in biotechnological applications microorganisms are frequently operated on the basis of pure culture systems which defines, but at the same time limits, the bioprocess operational space to the physiological boundaries of an organism [1]. As an alternative, utilization of microbial consortia allows inclusion of strains as contributors for enhancing bio-production by enabling eco-physiological functions and interactions through efficient utilisation of unrefined substrates [2,3], reducing by-product inhibition [4] and by performing eco-biotechnological optimization [5], to achieve high productivity and/or yield [6,7••].

Bioprocesses that are operated with self-selecting microbiomes, where the eco-physiological role and metabolic potential of individual microorganisms are not yet known and consequently not yet controllable, face certain difficulties. Some of the biggest challenges of using self-selecting microbiomes are maintaining the production beyond established metabolic and physiological boundaries for extended periods of time and promoting growth of specific organisms by sustaining their nutritional requirements [8]. Enriching specific phylogenetic groups of microorganisms might accelerate substrate uptake in self-selecting mixed culture applications. Moreover, microorganisms have evolved to proliferate as rapidly as possible, including to gain a maximum energy yield from a certain substrate [9]. However, substrate or feedback inhibition [10–12] of generated by-products, which are not necessarily intended products of a bioprocess, might also affect the bioprocessing characteristics. Even though the bioprocess parameters (e.g. pH, substrate concentration, feeds) are controlled, the metabolic interactions of microorganisms vastly remains an eco-physiological 'black box', and these unidentified interactions within the community limit streamlining the self-selecting mixed microbial ecosystem with regard to high productivities and yields for achieving superior productivity at an industrial scale [13].

Artificial microbial consortium (AMC) design and engineering has become an important avenue in biotechnology [14•]. As such, an AMC is regarded as part of the solution to debottleneck inherent physiological limitations of microbes of current bioprocesses that are operated with wild-type, metabolically engineered pure cultures, or self-selecting microbiomes [15]. AMC engineering can be considered as a sister approach to metabolic engineering [16]. It allows to broaden the pathway spectrum by integrating the metabolic capabilities of physiologically different species. Assuming that microbial pathways (including regulatory mechanisms etc.) are optimized within each organism, this AMC engineering possesses the potential to be more successful than squeezing desired pathways into a single microorganism. However,

an AMC engineering approach at optimum culture conditions of only one of the consortium members, which employs biotechnologically not well-characterized organisms, does not imply that the full potential for bio-production will be unleashed. Hence, AMC members must be physiologically well-selected to meet eco-biotechnological demands.

It has been suggested that for achieving higher production rates with an AMC approach, further engineering of the consortium should include regulation of the growth of the individual consortium members [17]. It has been also reported that the initial inoculation ratio needs to be considered as a key experimental parameter, since it has a vital impact on community structure, production and microbial interaction, and regulates the function of the consortium by inducing the desired properties [7••,18]. Therefore, a precision design strategy of an AMC should be employed to accomplish high efficiency and productivity in the targeted bioprocess. Here, we discuss a knowledge-based precision design strategy and show how an AMC engineering approach could be applied in the case of biohydrogen production.

Molecular hydrogen (H_2) holds great potential for generating sustainable energy at large scale and is regarded as a clean energy carrier of the future. Among the biohydrogen production methods, dark fermentative H_2 production (DFHP) has specific advantages over other production routes, such as high rate of cell growth, non-requirement of light energy, higher H_2 evolution rate (HER) [7••,19•,20]. However, a low yield of H_2 per consumed substrate ($Y_{(H2/S)}$) is imminent to using pure cultures or self-selecting microbiomes. Theoretically, DFHP is constrained to a maximum of 4 mol H_2 produced per one mol of glucose consumed when acetate is produced as a by-product [21]. This limitation constrains DFHP microorganisms in wild-type pure culture organisms and in self-selecting microbiomes. H_2 producing community structures were mainly explored in relation to specific processes such as maintenance of anoxic conditions (e.g. *Klebsiella* spp.) [22], substrate hydrolysis (e.g. *Bifidobacterium* spp.) [23], and inhibition of DFHP (e.g. *Lactobacillus* spp.) [24]. However, a lack of attention to the eco-biotechnological perspective could be considered as the main limiting factor for an advancement establishing H_2-producing microbial consortia with superior $Y_{(H2/S)}$ or HER [6,7••]. To overcome these bottlenecks, understanding consortia functioning with respect to optimized performance and further eco-biotechnological aspects have to be investigated, as well as potential interactions among the microbes and systemic properties of their organization should be examined [25].

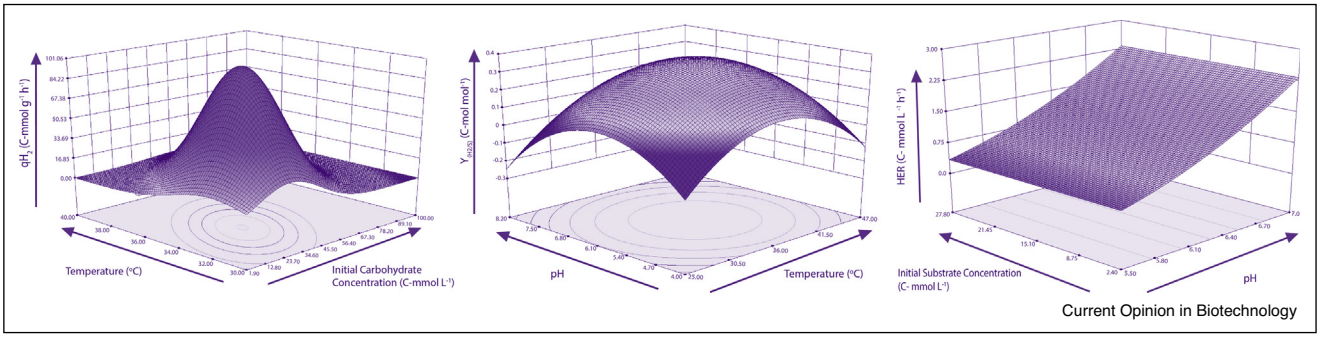
Design and engineering pipeline for developing DFHP bioprocesses

Before constructing an AMC of H_2 producing organisms, the industrial frame and requirements have to be considered. This frame and the needs shape the selection of microorganisms and this renders the bioprocessing conditions. The bioprocess conditions and the matching microorganisms are interdependent; accordingly, the selection stage might not be straightforward due to specific industrial requirements. Therefore, the selection of microorganisms must occur according to their bioprocess space, also referred to as ecological niche. Furthermore, the eco-biotechnological conditions (bioprocess parameters) for cultivating the individual pure cultures are key to engineering a synthetic consortium that exhibits high specific productivity (qH_2), $Y_{(H2/S)}$ and/or HER (Figure 1). Hence, the individual bioprocess conditions need to be aligned to the multivariate ecological niches of the microbes.

Knowledge-based physiological selection of microorganisms

Meta-data-analysis and subsequent multivariate statistical modelling of physiological properties of pure DFHP cultures are considered cornerstones that lead to the success of subsequent design and engineering of an

Figure 1



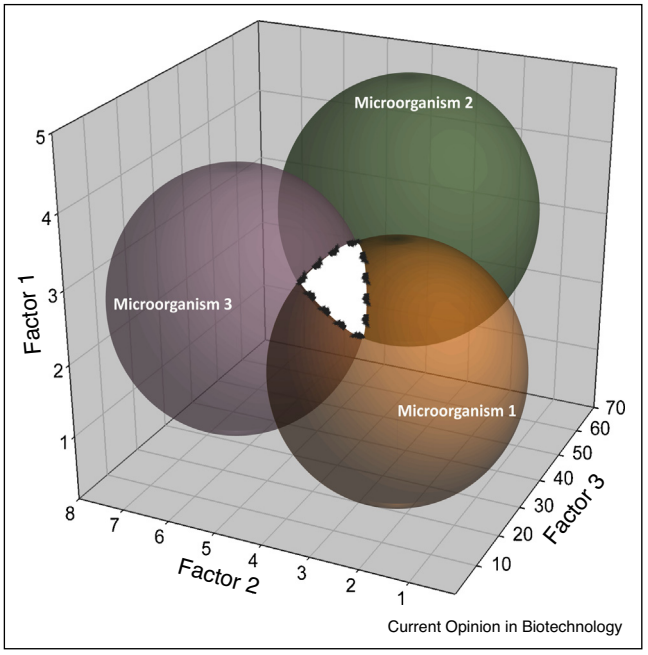
Exemplary results of meta-data analysis for physiology-based selection of microorganisms. The selection can be performed according to their multivariate bioprocess space where they exhibit high qH_2, $Y_{(H2/S)}$ and/or HER.

AMC [19•,26,27,28•]. With regard to self-selecting mixed culture DFHP, bioreactor operational conditions and the microbial composition of such consortia were analysed. The performance and stability of bioreactors containing self-selecting mixed microbial cultures and the species-level information for increasing $Y_{(H2/S)}$ were investigated. Moreover, the co-dependency between operational conditions, community structure and bioreactor functioning were examined, too. It is concluded that ecological and evolutionary process has to be considered to design high $Y_{(H2/S)}$ and long-term stable consortia [29]. This top-down approach is in contrast to a bottom-up approach that we recently employed [19•]. However, we anticipated that special emphasis should be given to pure cultures in order to overcome physiological restrictions of self-selecting mixed microbial consortia. Therefore, an in-depth meta-data analysis, statistics, and modelling of DFHP was performed before starting the artificial eco-system design, serving as a knowledge basis to reveal the bioprocess conditions on different taxonomic levels and to unambiguously report qH_2,$Y_{(H2/S)}$ and HER, of pure cultures [19•]. It must be noted that the major limiting factor for a successful meta-data analysis is the lack of information on qH_2, $Y_{(H2/S)}$ and HER for DFHP non-model organisms. Through this approach, the link between microbial physiology, eco-physiology and eco-biotechnology of DFHP could be subsequently formed. Hence, a meta-data analysis of DFHP provides models for the input parameters (eco-physiological perquisites) of selected organisms and renders the multivariate parameters space for the next stage of the pipeline.

Eco-physiological design of AMC

The next step on the avenue to engineer an AMC is to individually examine the multivariate parameter space in which the microorganisms exhibit a superior $Y_{(H2/S)}$ and HER and accordingly, to match the ecological niches of the individual microorganisms in which they exhibit optimum performance. To achieve that, investigating the effect of process key parameters (e.g. substrate concentration, medium compositions) on growth and gas production, as well as defining a mutual medium for the members of the consortium are important eco-physiological factors [7••]. It must be noted that meta-data analysis can identify the most promising organisms. However, that does not necessarily mean that the identified organisms perform best under cultivation conditions, which apply any non-optimized system. Therefore, parallel medium screening and multivariate-nutritional requirement analyses approaches may be beneficial for medium and/or strain prioritization [30]. The initial substrate concentration and the essential nutritional compounds of each of the members of the consortium have to be considered to prevent substrate limitation and/or inhibition of DFHP as well as to provide an optimum multivariate ecological niche (e.g. pH, temperature, salt concentration, trace elements, co-substrates, by-products) that accommodate the target organisms (Figure 2). The

Figure 2



Example of overlapping ecological niches for subsequent mutual medium design in a multivariate bioprocess space with the essential nutritional compounds (factors) of each of the members of the AMC.

syntrophic relationships within the AMC defines the DFHP efficiency [31], and the medium composition effect substrate uptake and production kinetics. Thus, this stage is crucial since it would allow high level process control and provide an essential eco-physiological outcome with regard to the syntrophic characteristics of the consortium members. The effect of initial substrate concentration on AMC has been investigated by creating statistical models. Based on the models, medium composition was altered to improve DFHP [32,33]. However, the essential nutritional compounds for each of the mono-cultures were *a priori* not individually identified. Hence, previous AMC studies unfortunately overlooked the necessity of an eco-physiological design stage as well as optimization of the mutual medium.

To be able to improve the DFHP, two major community function-determining parameters, initial substrate concentration and design of a mutual medium, should be individually and syntrophically investigated. Multivariate statistical design and optimization is regarded as the method of choice to match the multivariate ecological niches of the organisms in a straightforward way. In an AMC design and engineering study using *Enterobacter aerogenes* and *Clostridium acetobutylicum*, optimization of initial substrate concentration and design of a mutual medium where performed with the goal to attain the highest level of gas production occurred during growth of

Table 1

$Y_{(H2/S)}$ values of AMC grown on glucose in complex medium

Microorganisms	$Y_{(H2/S)}$/mol mol^{-1}	Temperature / °C	pH	Cultivation condition	Reference
Ruminococcus albus *Wolinella succinogenes*	3.91		6.8	Continuous	[45]
Caldicellulosiruptor saccharolyticus *Caldicellulosiruptor kristjanssonii*	3.7	70	6.7	Continuous	[36]
Clostridium sp. *Thermoanaerobacterium* sp.	0.8	60	6	Continuous	[46]
Klebsiella pneumoniae *Citrobacter freundii*	2.07	37	6.5	Batch	[47]
Clostridium butyricum *Clostridium pasteurianum*	2.12	30	5.3	Batch	[48]
Caldicellulosiruptor saccharolyticus *Caldicellulosiruptor owensensis*	4.42	70	6.5	Continuous	[44•]
Thermatoga neapolitana *Caldicellulosiruptor saccharolyticus*	2.8	75	7.0	Closed batch	[49]
Clostridium beijerinckii *Clostridium saccharoperbutylacetonicum*	2.00	37		Closed batch	[50]
Enterobacter cloacae *Bacillus cereus*	3.00	37	7.0	Batch	[38••]
Escherichia coli *Clostridium butyricum*	1.65	37		Closed batch	[40]
Enterobacter aerogenes *Clostridium acetobutyricum*	5.6	37	6.8	Closed batch	[7••]

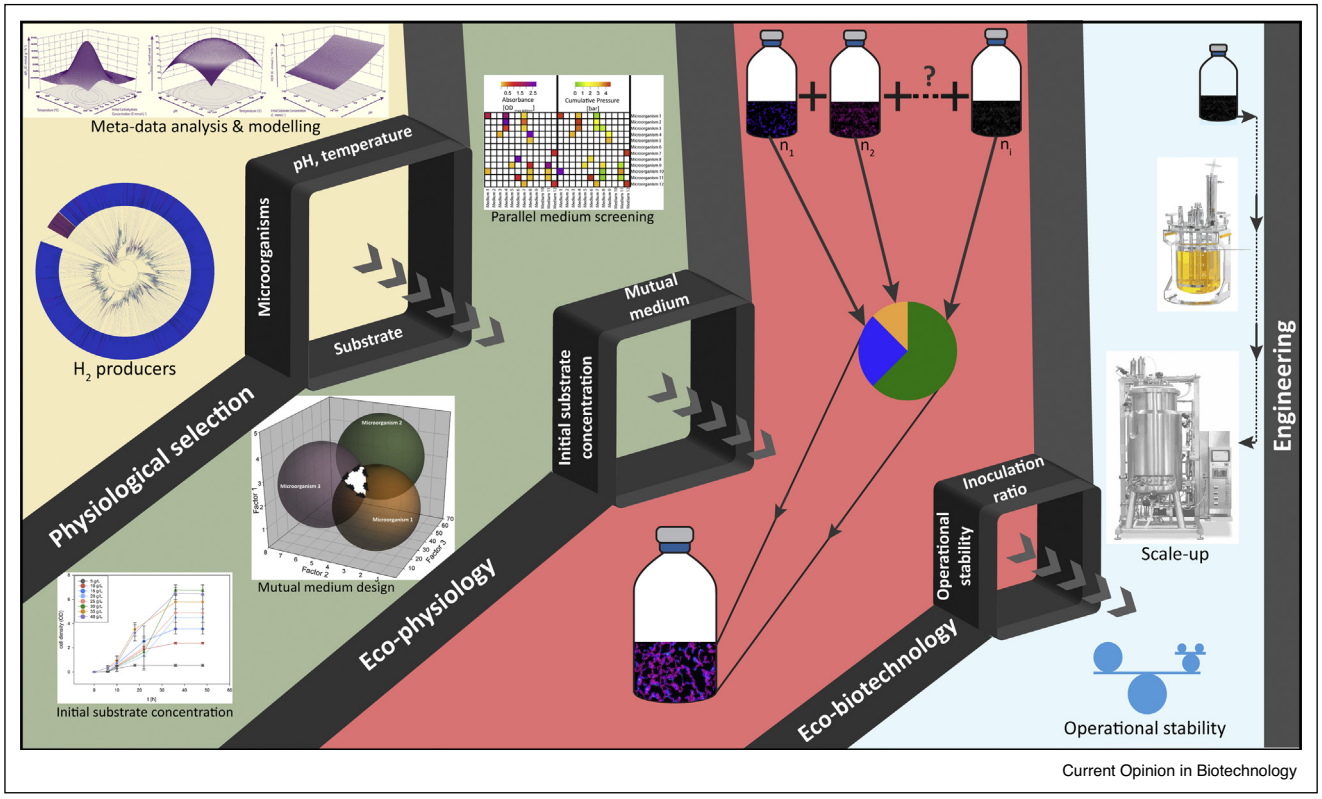
each strains individually [7••]. This design-based stage of the pipeline reveals the eco-physiological requirements for improving DFHP performance of the individual players in the community, and defines the required adjustments for a mutual environment.

Eco-biotechnological engineering of AMC

The last stage of the pipeline involves the AMC assembly, where the physiological, and eco-physiological prerequisites are integrated. Additional major community function-determining parameters with regard to AMC design and engineering are the ratio and the activity of initial cell densities of the community members. The ratio and activity of microorganism's impact $Y_{(H2/S)}$ and qH_2 of the individual community members due to substrate uptake, growth and production kinetics. It has been reported that the initial inoculation ratio of an AMC regulates the interactions between the microorganisms and metabolic capacity [34], and might have a major impact on improvement of DFHP [7••,31,35]. The majority of AMC studies were conducted with equal initial cell concentrations (1:2 ratio). Moreover, several studies reported that the lag phase of microorganisms become shortened and $Y_{(H2/S)}$ improved, compared to individual members alone under the same conditions, which was already pointing to a synergistic effect of the consortium [36,37,38••,39–41]. The effect of different inoculum ratios on DFHP has also been investigated [35,42,43], which concluded the activity and ratio of organisms in a consortium must be adjusted to reach higher efficiency. In this regard, a co-culture of *Clostridium thermopalmarium* and *Clostridium thermocellum* (0.05:1.05) provided an increase of DFHP in comparison to a pure culture of *C. thermocellum* on cellulose [42]. In conclusion, there are several studies that targeted modifying the activity and ratio of organisms resulting in an improvement of DFHP — however, the individual findings yet remained unconnected.

According to the meta-data of continuous culture pure culture DFHP, the best performing microbial families regardless of substrate type and cultivation conditions that we know to date are in the following order: Thermococcaceae, Clostridiaceae, Thermoanaerobacteriales Family III (IS), Ruminococcaceae, and Enterobacteriaceae [19•]. As shown in Table 1, the highest reported $Y_{(H2/S)}$ of an AMC study was 4.42 mol mol^{-1} on glucose, which was not the sole carbon source due to the usage of a complex medium, using a consortium containing *Caldicellulosiruptor saccharolyticus* and *Caldicellulosiruptor owensensis* from the Thermoanaerobacteriales Family III [44•]. However, an AMC of *E. aerogenes* and *C. acetobutylicum* with an inoculum ratio of 1:10 000 resulted a $Y_{(H2/S)}$ of 5.58 mol mol^{-1} in a glucose-containing chemically defined medium — a $Y_{(H2/S)}$ 40% higher than the Thauer limit. This might be due to a reduction in the excretion of ethanol and an increase in production of acetic acid and formic acid. The mono-culture of *E. aerogenes* shifts the metabolism from acid production to non-acid production below a pH of 5.8, which results in a decrease of H_2 production. However, the AMC is able to produce H_2 due to the activity of *C. acetobutylicum* at a pH range of 5.5 to 4.5. Moreover, H_2 production commences earlier in the AMC, and it exhibits a higher HER when a parallel exponential growth phase occurs. Eventually, the AMC

Figure 3



Overview of the proposed pipeline for AMC design and engineering. The pipeline starts with meta-data analysis of microbial physiologies for selection of microorganisms (left). Then medium screening and mutual medium design and optimization in the eco-physiological section may be performed. Thereafter, the eco-biotechnological characteristics of the culture should be examined and optimized. Finally, the scale-up and engineering may be performed.

displays a 6.6-fold higher $Y_{(H2/S)}$ compared to the monocultures and shows improved H_2 production kinetics compared to any system reported to date [7••].

Concluding remarks and outlook

- The proposed design and engineering pipeline (Figure 3), starting with a meta-data analysis of known DFHP physiologies to AMC engineering could be considered as a blueprint for improving other bioprocesses that inherently face metabolic restrictions or kinetic limitations.
- Knowledge-based strain selection and eco-physiological design of AMC streamlines subsequent multivariate eco-biotechnological optimization with regard to syntrophic performance characteristics of AMC.
- Identification of scale-up criteria and parameters in batch cultivation mode and long-term bioprocess stability of AMC in continuous culture are urgently required, as only few results from these key steps of bioprocess development are yet available.
- Further improvement of DFHP might be achieved by selecting microorganisms which exhibit higher qH_2 and $Y_{(H2/S)}$ (e.g. Thermococcaceae) keeping in mind that the high biomass concentrations are needed for attaining a high HER.
- Considering that Thermococcaceae were identified as the superior phylogenetic group for DFHP during the physiological assessment, this group of archaea could be of relevance for establishing AMC in the frame of Archaea Biotechnology.

Conflict of interest statement

Nothing declared.

CRediT authorship contribution statement

İpek Ergal: Conceptualization, Visualization, Writing - original draft, Writing - review & editing. **Günther Bochmann:** Funding acquisition, Project administration, Writing - review & editing. **Werner Fuchs:** Funding acquisition, Project administration, Writing - review & editing. **Simon K-MR Rittmann:** Conceptualization, Funding acquisition, Project administration, Writing - original draft, Writing - review & editing.

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References and recommended reading

Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

1. Grandel NE, Reyes Gamas K, Bennett MR: **Control of synthetic microbial consortia in time, space, and composition**. *Trends Microbiol* 2021 http://dx.doi.org/10.1016/j.tim.2021.04.001. in press.

2. Fu N, Peiris P, Markham J, Bavor J: **A novel co-culture process with *Zymomonas mobilis* and *Pichia stipitis* for efficient ethanol production on glucose/xylose mixtures**. *Enzyme Microb Technol* 2009, **45**:210-217.

3. Sun Y, Cheng J: **Hydrolysis of lignocellulosic materials for ethanol production: a review**. *Bioresour Technol* 2002, **83**:1-11.

4. Bhatia SK, Yi D-H, Kim Y-H, Kim H-J, Seo H-M, Lee J-H, Kim J-H, Jeon J-M, Jang K-S, Kim Y-G *et al.*: **Development of semi-synthetic microbial consortia of *Streptomyces coelicolor* for increased production of biodiesel (fatty acid methyl esters)**. *Fuel* 2015, **159**:189-196.

5. Lőrincz Z, Preininger É, Kósa A, Pónyi T, Nyitrai P, Sarkadi L, Kovács GM, Böddi B, Gyurján I: **Artificial tripartite symbiosis involving a green alga (Chlamydomonas), a bacterium (Azotobacter) and a fungus (Alternaria): morphological and physiological characterization**. *Folia Microbiol (Praha)* 2010, **55**:393-400.

6. Sabra W, Dietz D, Tjahjasari D, Zeng A-P: **Biosystems analysis and engineering of microbial consortia for industrial biotechnology**. *Eng Life Sci* 2010, **10**:407-421.

7. •• Ergal İ, Gräf O, Hasibar B, Steiner M, Vukotić S, Bochmann G, Fuchs W, Rittmann SK-MR: **Biohydrogen production beyond the Thauer limit by precision design of artificial microbial consortia**. *Commun Biol* 2020, **3**:443

In this study a precision design strategy of an AMC for DFHP is presented and an improvement of $Y_{(H2/S)}$ beyond the Thauer limit is shown.

8. Urbaniec K, Bakker RR: **Biomass residues as raw material for dark hydrogen fermentation – a review**. *Int J Hydrogen Energy* 2015, **40**:3648-3658.

9. Valdez-Vazquez I, Poggi-Varaldo HM: **Hydrogen production by fermentative consortia**. *Renew Sustain Energy Rev* 2009, **13**:1000-1013.

10. Vanniel E: **Distinctive properties of high hydrogen producing extreme thermophiles, *Caldicellulosiruptor saccharolyticus* and *Thermotoga elfii***. *Int J Hydrogen Energy* 2002, **27**:1391-1398.

11. Wang X, Ren N, Shengxiang W, Qianguo W: **Influence of gaseous end-products inhibition and nutrient limitations on the growth and hydrogen production by hydrogen-producing fermentative bacterial B49**. *Int J Hydrogen Energy* 2007, **32**:748-754.

12. Beckner M, Ivey ML, Phister TG: **Microbial contamination of fuel ethanol fermentations: bioethanol contamination**. *Lett Appl Microbiol* 2011, **53**:387-394.

13. Hardo G, Karunakaran E, Couto NA, Beckerman AP, Pandhal J: **Designing synthetic bacterial consortia for landfill leachate treatment based on community matrices and regression tree analysis**. *Microbiology* 2019 http://dx.doi.org/10.1101/543694v1.

14. • Diender M, Parera Olm I, Sousa DZ: **Synthetic co-cultures: novel avenues for bio-based processes**. *Curr Opin Biotechnol* 2021, **67**:72-79

A comprehensive overview of synthetic microbial co-cultivation studies to create bio-based processes with improved production characteristics.

15. McCarty NS, Ledesma-Amaro R: **Synthetic biology tools to engineer microbial communities for biotechnology**. *Trends Biotechnol* 2019, **37**:181-197.

16. Francois JM, Alkim C, Morin N: **Engineering microbial pathways for production of bio-based chemicals from lignocellulosic sugars: current status and perspectives**. *Biotechnol Biofuels* 2020, **13**:118.

17. Scholz SA, Graves I, Minty JJ, Lin XN: **Production of cellulosic organic acids via synthetic fungal consortia**. *Biotechnol Bioeng* 2018, **115**:1096-1100.

18. Gao C-H, Cao H, Cai P, Sørensen SJ: **The initial inoculation ratio regulates bacterial coculture interactions and metabolic capacity**. *ISME J* 2021, **15**:29-40.

19. • Ergal İ, Fuchs W, Hasibar B, Thallinger B, Bochmann G, Rittmann SK-MR: **The physiology and biotechnology of dark fermentative biohydrogen production**. *Biotechnol Adv* 2018, **36**:2165-2186

This is a comprehensive meta-data analysis and modelling study on DFHP. Strains, bioprocessing conditions, substrates, HER, $Y_{(H2/S)}$, qH_2 are reported and linked to microbial physiology and biotechnology.

20. Mauerhofer L-M, Pappenreiter P, Paulik C, Seifert AH, Bernacchi S, Rittmann SK-MR: **Methods for quantification of growth and productivity in anaerobic microbiology and biotechnology**. *Folia Microbiol (Praha)* 2019, **64**:321-360.

21. Thauer RK, Jungermann K, Decker K: **Energy conservation in chemotrophic anaerobic bacteria**. *Bacteriol Rev* 1977, **41**:100-180.

22. Hung C-H, Chang Y-T, Chang Y-J: **Roles of microorganisms other than *Clostridium* and *Enterobacter* in anaerobic fermentative biohydrogen production systems – a review**. *Bioresour Technol* 2011, **102**:8437-8444.

23. Weiland P: **Biogas production: current state and perspectives**. *Appl Microbiol Biotechnol* 2010, **85**:849-860.

24. Saraphirom P, Reungsang A: **Biological hydrogen production from sweet sorghum syrup by mixed cultures using an anaerobic sequencing batch reactor (ASBR)**. *Int J Hydrogen Energy* 2011, **36**:8765-8773.

25. Escalante AE, Rebolleda GÃ, BenÃtez M, Travisano M: **Ecological perspectives on synthetic biology: insights from microbial population biology**. *Front Microbiol* 2015, **6**.

26. Kushkevych I, Martínková K, Vítězová M, Rittmann SK-MR: **Intestinal microbiota and perspectives of the use of meta-analysis for comparison of ulcerative colitis studies**. *J Clin Med* 2021, **10**:462.

27. Rittmann SK-MR, Seifert AH, Bernacchi S: **Kinetics, multivariate statistical modelling, and physiology of CO_2-based biological methane production**. *Appl Energy* 2018, **216**:751-760.

28. • Gurevitch J, Koricheva J, Nakagawa S, Stewart G: **Meta-analysis and the science of research synthesis**. *Nature* 2018, **555**:175-182

A summary of accomplishments, limitations, recent advances and directions for future developments of meta-analysis and how meta-analysis could accelerate the progress in science by quantifying what is known to identify what is not yet known.

29. Navarro-Díaz M, Valdez-Vazquez I, Escalante AE: **Ecological perspectives of hydrogen fermentation by microbial consortia: what we have learned and the way forward**. *Int J Hydrogen Energy* 2016, **41**:17297-17308.

30. Mauerhofer L-M, Zwirtmayr S, Pappenreiter P, Bernacchi S, Seifert AH, Reischl B, Schmider T, Taubner R-S, Paulik C, Rittmann SK-MR: **Hyperthermophilic methanogenic archaea act as high-pressure CH_4 cell factories**. *Commun Biol* 2021, **4**:289.

31. Laxman Pachapur V, Jyoti Sarma S, Kaur Brar S, Le Bihan Y, Ricardo Soccol C, Buelna G, Verma M: **Co-culture strategies for increased biohydrogen production: strategies for increased**

biohydrogen production by co-culture system. *Int J Energy Res* 2015, **39**:1479-1504.

32. Pachapur VL, Sarma SJ, Brar SK, Le Bihan Y, Buelna G, Verma M: **Biohydrogen production by co-fermentation of crude glycerol and apple pomace hydrolysate using co-culture of *Enterobacter aerogenes* and *Clostridium butyricum***. *Bioresour Technol* 2015, **193**:297-306.

33. Phowan P, Reungsang A, Danvirutai P: **Bio-hydrogen production from cassava pulp hydrolysate using co-culture of *Clostridium butyricum* and *Enterobacter aerogenes***. *Biotechnology (Faisalabad)* 2010, **9**:348-354.

34. Gao C-H, Cao H, Cai P, Sørensen SJ: **The initial inoculation ratio regulates bacterial coculture interactions and metabolic capacity**. *ISME J* 2021, **15**:29-40.

35. Pachapur VL, Sarma SJ, Brar SK, Bihan YL, Buelna G, Verma M: **Hydrogen production from biodiesel industry waste by using a co-culture of *Enterobacter aerogenes* and *Clostridium butyricum***. *Biofuels* 2017, **8**:651-662.

36. Zeidan AA, van Niel EWJ: **A quantitative analysis of hydrogen production efficiency of the extreme thermophile *Caldicellulosiruptor owensensis* OLT**. *Int J Hydrogen Energy* 2010, **35**:1128-1137.

37. Zeidan AA, Van Niel EWJ: **Developing a thermophilic hydrogen-producing co-culture for efficient utilization of mixed sugars**. *Int J Hydrogen Energy* 2009, **34**:4524-4528.

38. •• Patel SKS, Kumar P, Mehariya S, Purohit HJ, Lee J-K, Kalia VC: **Enhancement in hydrogen production by co-cultures of *Bacillus* and *Enterobacter***. *Int J Hydrogen Energy* 2014, **39**:14663-14668

An excellent study on enhancement of the efficiency of DFHP by using defined co-cultures and their scale-up.

39. Cheng J, Zhu M: **A novel anaerobic co-culture system for bio-hydrogen production from sugarcane bagasse**. *Bioresour Technol* 2013, **144**:623-631.

40. Seppälä JJ, Puhakka JA, Yli-Harja O, Karp MT, Santala V: **Fermentative hydrogen production by *Clostridium butyricum* and *Escherichia coli* in pure and cocultures**. *Int J Hydrogen Energy* 2011, **36**:10701-10708.

41. Bao H, Chen C, Jiang L, Liu Y, Shen M, Liu W, Wang A: **Optimization of key factors affecting biohydrogen production from microcrystalline cellulose by the co-culture of *Clostridium acetobutylicum* X_9 + *Ethanoigenens harbinense* B_2**. *RSC Adv* 2016, **6**:3421-3427.

42. Geng A, He Y, Qian C, Yan X, Zhou Z: **Effect of key factors on hydrogen production from cellulose in a co-culture of *Clostridium thermocellum* and *Clostridium thermopalmarium***. *Bioresour Technol* 2010, **101**:4029-4033.

43. Chang J-J, Chou C-H, Ho C-Y, Chen W-E, Lay J-J, Huang C-C: **Syntrophic co-culture of aerobic *Bacillus* and anaerobic *Clostridium* for bio-fuels and bio-hydrogen production**. *Int J Hydrogen Energy* 2008, **33**:5137-5146.

44. • Pawar SS, Vongkumpeang T, Grey C, van Niel E: **Biofilm formation by designed co-cultures of *Caldicellulosiruptor* species as a means to improve hydrogen productivity**. *Biotechnol Biofuels* 2015, **8**:19

This study reports an improvement of substrate conversion and HER by using a co-culture of different *Caldicellulosiruptor* species in a continuous upflow anaerobic reactor.

45. Iannotti EL, Kafkewitz D, Wolin MJ, Bryant MP: **Glucose fermentation products in *Ruminococcus albus* grown in continuous culture with *Vibrio succinogenes*: changes caused by interspecies transfer of H_2**. *J Bacteriol* 1973, **114**:1231-1240.

46. Koskinen PEP, Beck SR, Örlygsson J, Puhakka JA: **Ethanol and hydrogen production by two thermophilic, anaerobic bacteria isolated from Icelandic geothermal areas**. *Biotechnol Bioeng* 2008, **101**:679-690.

47. Mishra P, Roy S, Das D: **Comparative evaluation of the hydrogen production by mixed consortium, synthetic co-culture and pure culture using distillery effluent**. *Bioresour Technol* 2015, **198**:593-602.

48. Masset J, Calusinska M, Hamilton C, Hiligsmann S, Joris B, Wilmotte A, Thonart P: **Fermentative hydrogen production from glucose and starch using pure strains and artificial co-cultures of *Clostridium* spp**. *Biotechnol Biofuels* 2012, **5**:35.

49. Okonkwo O, Lakaniemi A-M, Santala V, Karp M, Mangayil R: **Quantitative real-time PCR monitoring dynamics of *Thermotoga neapolitana* in synthetic co-culture for biohydrogen production**. *Int J Hydrogen Energy* 2018, **43**:3133-3141.

50. Nasr N, Gupta M, Hafez H, El Naggar MH, Nakhla G: **Mono- and co-substrate utilization kinetics using mono- and co-culture of *Clostridium beijerinckii* and *Clostridium saccharoperbutylacetonicum***. *Bioresour Technol* 2017, **241**:152-160.