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DOI

[10.1016/j.copbio.2021.01.016](https://doi.org/10.1016/j.copbio.2021.01.016)

Publication date

2021

Document Version

Final published version

Published in

Current Opinion in Biotechnology

Citation (APA)

Moscoviz, R., Kleerebezem, R., & Rombouts, J. L. (2021). Directing carbohydrates toward ethanol using mesophilic microbial communities. *Current Opinion in Biotechnology*, 67, 175-183.
<https://doi.org/10.1016/j.copbio.2021.01.016>

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Directing carbohydrates toward ethanol using mesophilic microbial communities

Roman Moscoviz¹, Robbert Kleerebezem² and Julius Laurens Rombouts²

Bioethanol production is an established biotechnological process. Margins are low which prevent a larger scale production of bioethanol. As a large part of the production cost is due to the feedstock, the use of low value unsterile feedstocks fermented by microbial communities will enable a more cost-competitive bioethanol production. To select for high yield ethanol producing communities, three selective conditions are proposed: acid washing of the cells after fermentation, a low pH (<5) during the fermentation and microaerobiosis at the start of the fermentation. Ethanol producers, such as *Zymomonas* species and yeasts, compete for carbohydrates with volatile fatty acid and lactic acid producing bacteria. Creating effective consortia of lactic acid bacteria and homo-ethanol producers at low pH will lead to robust and competitive ethanol yields and titres. A conceptual design of an ecology-based bioethanol production process is proposed using food waste to produce bioethanol, electricity, digestate and heat.

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Current Opinion in Biotechnology 2021, **67**:175–183

This review comes from a themed issue on **Environmental biotechnology**

Edited by **Robbert Kleerebezem** and **Diana Machado de Sousa**

<https://doi.org/10.1016/j.copbio.2021.01.016>

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Introduction

Ethanol production through biotechnological fermentation processes is an established industry. The global fuel ethanol market was estimated to be 110 billion litres in 2018 [1]. In the period of 2012–2019, the ethanol price in Europe, Brazil and the United States fluctuated between 0.95 and 0.28 USD per litre (0.79–0.25 € per litre) and prices have decreased from 2012 onwards, leading to a less

profitable market situation [2]. A large cost contributor to production of bioethanol is the feedstock. An European Commission report from 2002 estimated that 48–60% of the cost in ethanol production was due to the feedstock (in a case study described for sugar beets), with a total production cost of 0.42–0.54 € per litre [3]. Margins in the bio-ethanol industry are low and, in many countries, subsidies and mandatory blending are main drivers to sustain ethanol production [2].

Using less expensive feedstocks for fermentation can enable cost-competitive bio-ethanol production. First generation feedstocks used are carbohydrates from sugarcane, maize or sugar beets (first generation). Second-generation (i.e. non-food lignocellulosic feedstocks) and third generation (i.e. algae as feedstock) ethanol production are scaling up [1]. However, first-generation ethanol remains largely dominant on the market and represents more than 97% of the total bioethanol production [1]. In the last decade, a great deal of research has been dedicated to the use of lignocellulosic materials and microalgae for bioethanol production [4,5]. As these feedstocks and their pre-treatment to release fermentable sugars are relatively expensive [3], other substrates of interest have emerged, such as the organic fraction of municipal solid waste (OFMSW). Specifically, food and kitchen waste are of interest, as they are relatively inexpensive, abundant and rich in readily biodegradable carbohydrates [6,7]. Although food waste collection and sorting remain a challenge in many countries, such feedstocks have the potential to enable a more cost competitive bioprocess, if the ethanol yields and product concentrations (titres) per carbohydrate are sufficient.

It is predicted that 2.5 billion tonnes of food waste are going to be generated by 2025 annually worldwide [8]. In Europe, North America and Oceania, a one third of the food waste corresponds to restaurant or household waste which may contain animal by-products [9]. In that case, sanitary regulations in places such as the European Union limit the scope of food waste valorisation (e.g. no utilization as feed for animals) [10]. Thus, on a global scale food waste is generally landfilled [8] and if treated, it is mostly valorised by composting and/or biogas production [8].

Using food waste as feedstock for conversion towards higher value products such as bioethanol comes with some challenges since it is generally heterogeneous [7] and contains a high load of fermentative microorganisms

[11]. The use of pure cultures to ferment food waste to ethanol requires sterilization of the feedstock and large inocula (10%–15% v:v) [12]. Sterilization is challenging as either filtering, heating or UV sterilization will add to the costs and involve technological challenges. For example, thermal sterilization leads to loss of sugars due to Maillard reactions [13], while microfiltration of food waste leads to a loss of at least 25% of the fermentable sugars [12]. In 2007, Kleerebezem and van Loosdrecht proposed to exclude feedstock sterilization and use mixed-culture biotechnology or open microbial communities to circumvent these challenges [14]. In line with this work, Holtzapfel and Granda formulated the carboxylate platform as a way to efficiently transform biomass into alcohols like ethanol, using mixed cultures to produce acetate and a chemical reduction to produce ethanol from acetate using hydrogen [15]. Mixed culture-based processes do not require substrate sterilization and, due to microbial diversity, often display high adaptive capacity regarding substrate quality and environmental conditions. Van Loosdrecht and Kleerebezem commented that no selective conditions for bioethanol production were experimentally identified in 2007 [14]. Since then, novel insights have been described that suggest specific operational strategies that allow for effective enrichment of carbohydrate fermentation to ethanol. The aim of this article is to explore these ecology-based strategies enabling bioethanol production from carbohydrates to pave the way for efficient and competitive biorefinery approaches. Furthermore, a case study of food waste valorisation to bioethanol and electricity in the European Union is proposed as an example of a biorefinery efficiently utilising the ecology-based strategies for ethanol production.

A diversity of competing carbohydrate fermentation pathways

One of the main challenges of open mixed culture processes is product selectivity. Since microbial communities are diverse, many different metabolic pathways can be carried out in parallel, leading to a variety of fermentative products. Under anaerobiosis, ethanol is usually produced from carbohydrates through three types of fermentative pathways (Figure 1). Homo-ethanol production (pathway 1) leads to the highest theoretical yield of $0.51 \text{ g}_{\text{ethanol}} \text{ g}_{\text{glucose}}^{-1}$ and is commonly observed to be carried out by yeasts or the bacterium *Zymomonas mobilis* [16]. The two other pathways lead to the formation of ethanol together with lactate (heterofermentation, pathway 2) or with acetate and formate or hydrogen (acetate and ethanol production, pathway 3). The latter two catabolic pathways leading to a theoretical yield of $0.26 \text{ g}_{\text{ethanol}} \text{ g}_{\text{glucose}}^{-1}$. Heterofermentation is described for lactic acid bacteria [16] while acetate and ethanol production has been linked to both lactic acid bacteria and *Enterobacteriaceae* species, such as *Escherichia* or *Klebsiella* species [16]. These three pathways are also in direct competition for carbohydrates with other pathways

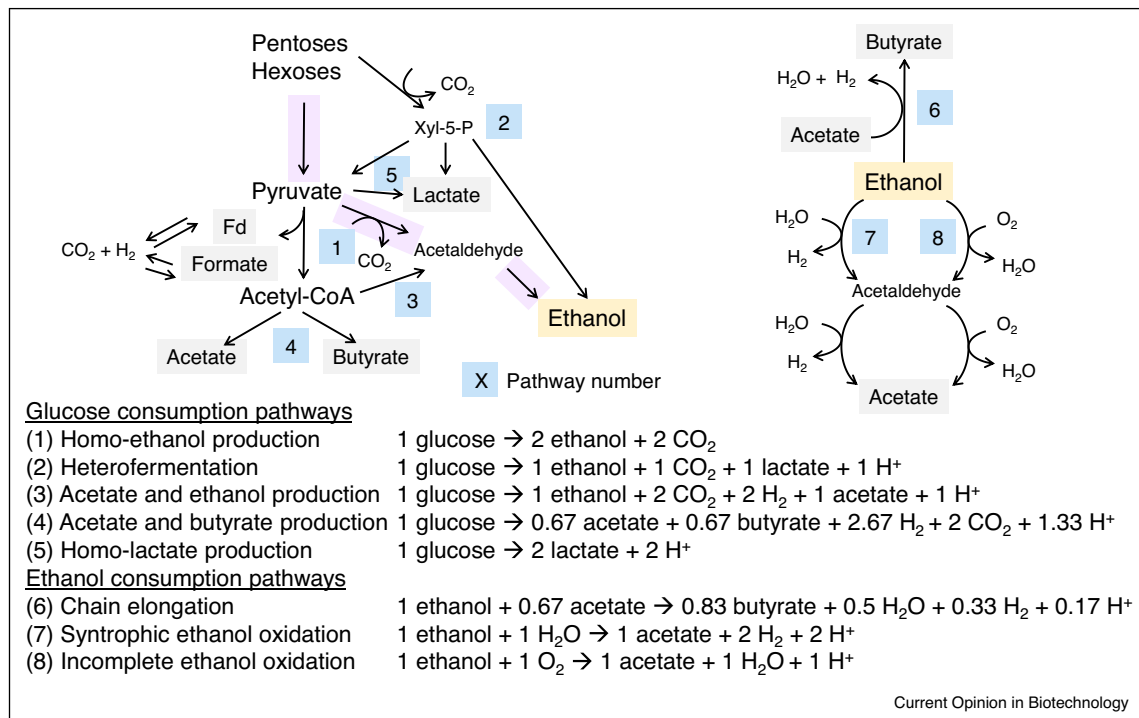
yielding volatile fatty acids such as acetate and butyrate (pathway 4) [17], often linked to *Clostridium* species [16], or solely lactate production by lactic acid bacteria (pathway 5) [16]. Ethanol can also be consumed in anaerobic environments as electron donor for chain elongation of volatile fatty acids by species such as *Clostridium kluyveri* (pathway 6) [18], or oxidized anaerobically to acetate and H_2 through syntrophic ethanol oxidation (pathway 7) [19]. In the presence of oxygen ethanol can be converted to acetate through incomplete ethanol oxidation (pathway 8), which has been linked to *Acetobacter* and *Gluconobacter* species [16].

The goal of mixed-culture bioethanol production is to impose the right selective conditions that maximise the carbon conversion from carbohydrates towards ethanol, while avoiding ethanol consumption. Ideally, homo-ethanol production is maximised. However, only few studies have focused on mixed-culture biotechnology for bioethanol production and such selective conditions are not yet well documented as reviewed recently [20*]. In this work we will propose ecological strategies that lead to high ethanol production. These strategies are mainly based on the abundant literature available on dark fermentation [20*], the analysis of spontaneous ethanol fermentation used in traditional beverage production, physiological studies of known fermentative organisms and microbial contaminations of pure culture bioethanol production processes.

Acid wash during cell recycling and low pH fermentation enhances ethanol production

Early modelling work by Rodriguez *et al.* [21] predicted that low pH (<5.6) should theoretically favour ethanol production instead of organic acids, since acid transport outside cells becomes energetically very expensive when the extracellular pH is close to the pK_a value of these acids (around 4.8). This trend seems to be experimentally verified by the literature on dark fermentation (see Supplementary material). A meta-analysis of 150 mesophilic mixed-culture dark fermentations of carbohydrates showed that ethanol yields were significantly higher when pH was below 5 when compared to pH between 5 and 6 (p-value <0.01) or between 6 and 7 (p-value <0.05). However, ethanol yields and titres remained low in these studies ($<0.15 \text{ g}_{\text{EtOH}} \text{ g}_{\text{glucose-eq}}^{-1}$ and 5 g L^{-1} , respectively). This result is likely due to the primary focus of these studies (i.e. H_2 production optimization) and the use of inoculum heat treatment (usually 90–100°C between 15–30 min) in more than 70% of the cases. This heat treatment is carried out to remove non-spore forming bacteria, such as the homo-ethanol producing *Zymomonas* species and to promote spore forming bacteria such as *Firmicutes* species which produce high yields of hydrogen. This heat treatment is also lethal to fungi, including yeasts [22]. Microbial communities in these studies were likely highly dominated by heat-shock resistant bacteria, such as *Clostridium* species [23]. *Clostridium* species do not

Figure 1



Simplified metabolic pathways yielding dominant fermentative products from carbohydrates and simplified metabolic pathways consuming ethanol. The homo-ethanol pathway (1) is highlighted in purple. Xyl-5-p = xylulose-5-phosphate, Fd = ferredoxin. Based on [16]. Pentoses can be xylose, arabinose and so on. Hexoses can be glucose, galactose, fructose and so on.

harbour the pyruvate decarboxylase gene to go from pyruvate to acetaldehyde (Figure 1, search in GenBank of NCBI 23rd Nov 2020) and therefore do not utilise the homo-ethanol pathway.

Yeasts can thrive at low pH and usually dominate during spontaneous ethanol producing fermentations where pH is typically below 4, such as grape juice fermentation for wine production [24], barley fermentation for Belgian sour ales [25,26**] and milk fermentation for kefir [27,28]. Peinemann *et al.* recently carried out a non-sterile food waste fermentation inoculated with *Saccharomyces cerevisiae* [29**]. When *S. cerevisiae* competed against the native food waste microbial community, the authors obtained ethanol yields and titres of 0.33 g_{EtOH} g_{glucose-eq.}⁻¹ and 41 g L⁻¹, respectively. When *S. cerevisiae* was co-inoculated with a lactic acid bacteria-dominated mixed culture, they observed that ethanol yields and titres remained competitive at low pH (uncontrolled, around 4), with values of 0.36 g_{EtOH} g_{glucose-eq.}⁻¹ and 45 g L⁻¹, respectively. However, when pH was regulated at six, ethanol yields and titres were reduced twofold (0.15 g_{EtOH} g_{glucose-eq.}⁻¹ and 19 g L⁻¹, respectively) while lactate became the dominant product. Yeasts are also shown to remain viable after prolonged cycles of acid treatment at pH 2.0 [30] and are shown to retain a similar

cell viability after 120 min of acid treatment [31]. Gibson *et al.* proposed that one of the selective conditions for yeasts to dominate a microbial community and cause ethanol production is a very low pH biomass-wash step during cell recycling [32*]. In fact, this practice is commonly used in brewing industries and bioethanol producing facilities to inhibit bacterial growth [32*,33*]. However, lactic acid bacteria can tolerate very low pH (below 3). For instance, *Lactobacillus plantarum* showed 50–100% cell survival after 30 min at pH 2 [34]. Introducing an acid wash step when recycling cells after the fermentation will promote yeasts and thereby increase the ethanol yield in fermentation.

As the meta-analysis points out, low pH fermentation (pH below 5) seems to be beneficial for ethanol production. *Clostridium* species responsible for acetate and butyrate production are less competitive in environments with low pH as the production of kefir and sour ales points out [25,26**,27,28]. At low pH, ethanol scavengers are anticipated to be completely inhibited, as chain elongating organisms and syntrophic ethanol oxidizers cannot cope with such a low pH [18,35]. *Enterobacteriaceae* will likely be outcompeted at low pH since they were not observed in anaerobic glucose

fermenting batch environments [36^{••}], and neither were identified as bacterial contaminant in bioethanol production plants [37]. Furthermore, *Zymomonas mobilis* ferments actively down to pH 3.5 [38], and thus is able to co-ferment with yeasts in a low pH environment. Microbial community analyses of industrial bio-ethanol production contaminants confirmed the presence of *Zymomonas* species in the fermentation stage [37]. Lactic acid bacteria are competitive at pH values below 5 using mainly heterofermentation as dominant pathway instead of homolactic acid production [36^{••},39^{••}].

In summary, we propose that maintaining a low pH (<5) during fermentation will prevent ethanol consumption and favour ethanol producing pathways over volatile fatty acid producing routes. Still, the production of lactic acid as side-product may lower the ethanol yield in the process.

Initial microaerobiosis to stimulate yeast growth

Oxygen can be used to inhibit the growth of microbial groups responsible for low ethanol yields. For instance, *Clostridium* species carrying out chain elongation and syntrophic ethanol oxidizers are obligate anaerobes and strictly inhibited in the presence of oxygen [16]. Lactic acid bacteria can tolerate oxygen and *Enterobacteriaceae* are facultative anaerobes [16], thus they will not be inhibited by introducing oxygen. Incomplete ethanol oxidation can occur during oxygen presence, but at pH 4 or lower this activity is likely not competitive [40].

Conversely, oxygen availability favours the growth of yeasts. A comparative study showed that only 23% of the type species for 75 yeast genera were in fact able to grow anaerobically [41]. Of all these species, *S. cerevisiae* was observed the most competitive fermenter in anaerobic conditions [41], showing a μ of 0.40 h^{-1} in batch. *S. cerevisiae* showed little difference in growth rate between aerobic and anaerobic conditions [41]. Early research has shown that *S. cerevisiae* grows only anaerobically when ergosterol and unsaturated fatty acids are supplied [42]. Metabolic network models suggest that ergosterol and oleic acid production require oxygen [43]. Recent work has shown anaerobic growth of *S. cerevisiae* is possible without oxygen supplementation [44], though cell viability under very low pH (1.5) and high ethanol titres (100 g L^{-1}) was only retained when oxygen was available for the yeast cells.

However, oxygen availability will likely stimulate reducing sugar consumption through aerobic respiration and thus compete with ethanol production. Therefore, oxygen presence must be limited to the initial stage of fermentation to limit respiration and growth of unwanted

bacterial species while providing a competitive advantage to yeasts and oxygen-tolerant species.

Effective consortia of lactic acid bacteria and yeasts

Lactic acid bacteria often share the same environmental niche as yeasts since they are both tolerant to low pH and high ethanol concentrations. For example, the maximum growth rate of *Lactobacillus plantarum* was reduced by only 57% when grown in presence of 63 g L^{-1} ethanol. *Lactobacillus heterohiochii* only started to be affected at 100 g L^{-1} of ethanol [45,46]. These species are regularly found as bacterial contamination in industrial pure culture yeast fermentations [47]. For instance, Rich *et al.* investigated the bacterial contamination of five commercial corn-based ethanol production processes and found that *Lactobacillus* species were systematically the dominant bacterial contaminant [37]. These contaminants have been reported to reduce ethanol yields in industrial processes by up to 30% by direct competition for substrate, production of inhibitory metabolites (e.g. acetic acid), and also cause several hazards of biofilm and foam formation [39^{••},47].

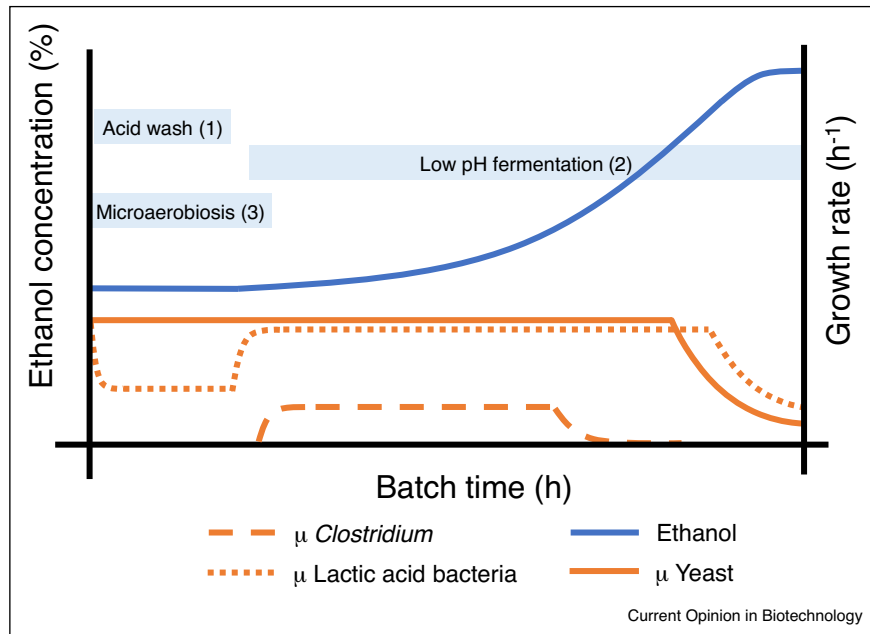
Industrial pure culture processes are susceptible to contaminations as compared to selected ecologically stable microbial communities. Not all lactic acid bacteria are detrimental to ethanol production [39^{••}] and some may even help protecting the fermentative community against external contaminants (e.g. by secreting bacteriocins). For instance, Rich *et al.* carried out tricultures of *S. cerevisiae*, *Lactobacillus fermentum* (detrimental for ethanol production) and a third species selected from over 500 lactic acid bacteria isolated from industrial fuel ethanol fermentations [48[•]]. The authors found that over 300 isolates were able to partially or totally restore ethanol production to the levels obtained by pure culture of *S. cerevisiae*. Other bacterial species may also help preventing biofilm formation by lactic acid bacteria. For example, several *Bacillus* spp. were found to excrete compounds which did not affect yeast growth but limited biofilm formation by common *Lactobacillus* species such as *Lactobacillus fermentum*, *L. plantarum* and *L. brevis* [49].

Based on these considerations, we propose that stable microbial communities of lactic acid bacteria and ethanol producers, when grown in a selective environment that favours ethanol production, will likely act synergistically and yield high ethanol titres and productivities.

Designing novel bioprocesses: a research outlook

By using both pH strategies and initial microaerobiosis, we propose that it is possible to effectively enrich and maintain an ethanol-producing microbial community (Figure 2). A first option would be to create an effective microbial community by inoculating an efficient pure

Figure 2

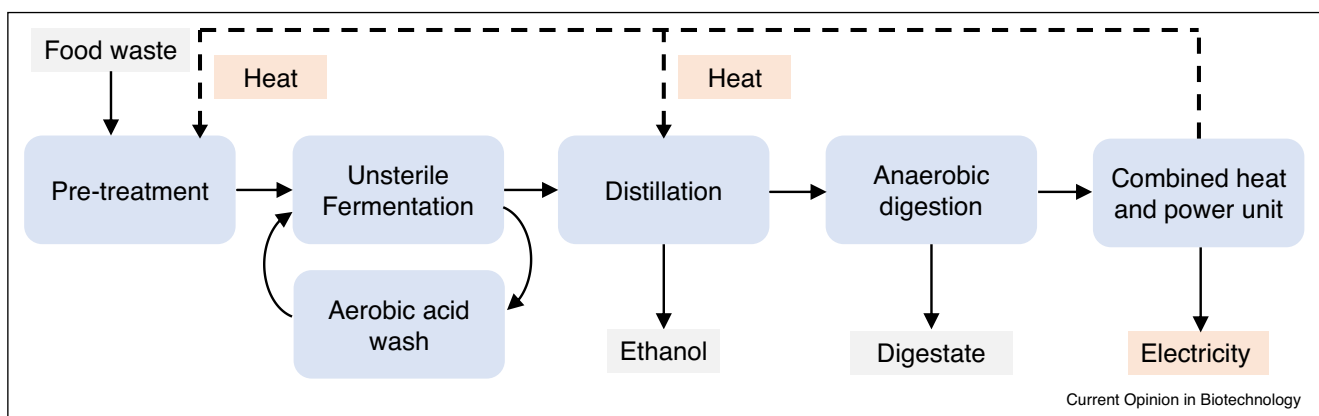


The three operational measures and the hypothetical effect on the growth rate of different groups of carbohydrate fermenting microorganisms that are proposed to favour carbohydrate fermentation to ethanol using an ecology-based design. Yeasts, lactic acid bacteria and *Clostridium* are considered the most competitive fermentative organisms in this environment and are therefore visualised. A new feed is introduced at the end of the acid wash phase. Growth rates (μ) are affected by all three selective conditions, which are present during the length of the box of the batch fermentation.

strain (e.g. *S. cerevisiae*) in the unsterile fermentation medium, as carried out by Peinemann *et al.* [29]. The resulting mixed culture would then be reused in the next fermentations without the need for pure cultivation. Another option would consist in orienting a complex mixed culture into an efficient ethanol-producing consortium. This second strategy has only been demonstrated

twice so far using low concentration of glucose as substrate. Tamis *et al.* (paper submitted for publication) have observed the enrichment of a mixture of yeasts (*Candida* and *Pichia*) and lactic acid bacteria (*Lactobacillus*) in a sequencing batch granular biomass fermentation process operated at pH 4 and 3.5, where the ethanol yield increased from 0.28 to 0.31 g_{EtOH} g_{glucose}⁻¹ with

Figure 3



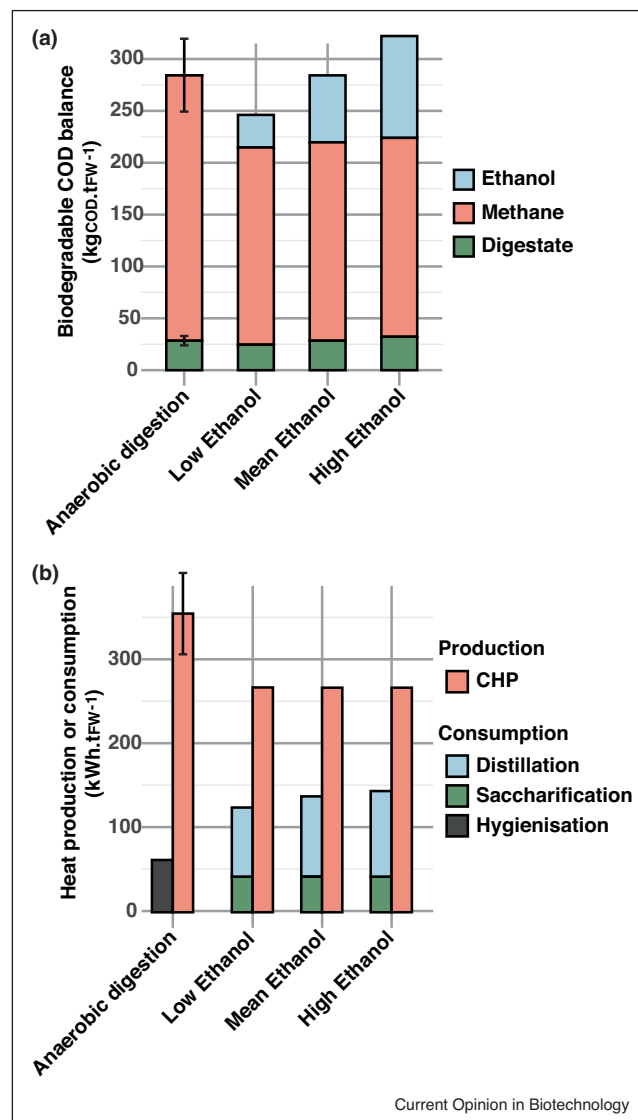
A scheme for producing bioethanol from food waste in energy efficient process by integrating the excess heat from the combined heat and power unit to distillation. Process stages are blue, main materials streams are in grey boxes, energy streams in orange.

decreasing pH (maximum titre of 3.1 g L^{-1}) [50]. Darwin *et al.* have shown a yield of up to $0.38 \text{ g}_{\text{EtOH}} \text{ g}_{\text{glucose}}^{-1}$ (titre of 15.2 g L^{-1}) in a continuous process operated at a pH of around 3.0 [51**], showing homo-ethanol production can outcompete heterofermentation.

Mixed culture-based bioethanol production processes would reach lower ethanol yields and thus titres, when compared to their pure culture equivalent, with yields of about $0.38 \text{ g}_{\text{EtOH}} \text{ g}_{\text{glucose-cq}}^{-1}$ [51**] instead of $0.45 \text{ g}_{\text{EtOH}} \text{ g}_{\text{glucose-cq}}^{-1}$ [52]. However, these processes can be designed in an effective biorefinery framework where ethanol is one of the final products. One of such a process could be designed as follows (Figure 3): (i) food waste is pre-treated for hydrolysis of particulate substrates such as starch and cellulose; (ii) carbohydrates are fermented to ethanol by a mixed microbial consortium; (iii) ethanol is recovered from the fermentation broth via distillation; and (iv) the remaining still bottom is digested to biogas that (v) is converted to heat and electricity using a combined heat and power (CHP) plant. Pre-treatment can be designed in a cost-competitive way using a fungal mash produced with a small part of the food waste, containing starch and cellulose hydrolysing enzymes [12]. Alternatively, a more classic approach involving enzyme addition can be considered [53,54]. A high glucose concentration before fermentation is then created, which benefits yeasts and thus ethanol production [51**]. The residual heat from the CHP can be transferred back to the pre-treatment and distillation to create a cost and energy efficient process. An example of such an integrated process has been proposed by Bouchez and Richard [55], though no clear ethanol promoting selection pressure is proposed for the fermentation stage.

Using data from the literature, a simplified mass and heat balance of such process was calculated and compared to a scenario where all food waste was directly used as anaerobic digestion feedstock (see Figure 4 and Supplementary material). For an average food waste ($24.5 \pm 4.5\%$ TS) [7], depending on carbohydrate content ($58 \pm 7\%$ TS), saccharification (about $0.75 \text{ g}_{\text{glucose-cq}} \text{ g}_{\text{carbohydrate}}^{-1}$) and ethanol production yields (0.28 to $0.38 \text{ g}_{\text{glucose-cq}}^{-1}$, ethanol concentrations between 21 and 59 g L^{-1}) were estimated. These concentrations are largely lower than what is usually attained in first generation ethanol fermentation (around 120 g L^{-1}), leading to a low energy efficiency during the distillation step (Figure 4b). However, heat production from CHP (e.g. 110°C stream) can cover thermal requirements for distillation in all cases, thus showing the potential synergy between ethanol production and anaerobic digestion. It must also be mentioned that the distillation step will also ensure food waste hygienisation, a mandatory step in the EU for household or restaurant waste containing animal by-products [10] Assuming that all 129 million tonnes kitchen waste generated in the EU in 2011 [56] was valorised

Figure 4



Mass (a) and heat (b) balances of an ethanol food waste fermentation process coupled with anaerobic digestion. The Anaerobic digestion scenario is provided as reference and corresponds to a direct mesophilic conversion of food waste into methane, followed by heat and electricity production. Low, Mean and High ethanol correspond to pessimistic, average, and optimistic scenarios, respectively, for ethanol production coupled to anaerobic digestion. These three scenarios follow the scheme presented in Figure 3, saccharification being used as pre-treatment. Differences between the three scenarios lie in the food waste concentration [7], carbohydrate content [7], saccharification [53,54] and ethanol [51**] production yields. The detailed calculation is provided in Supplementary material.

through this process, about 4 million tonnes of bioethanol can be produced through this process, which represents 86% of the EU market in 2019 [2]. Yet, more detailed techno-economic studies would be required to optimize the process configurations. Alternatively, in cases where

the distillation step is too expensive due to low ethanol titres, fermentation broth containing mainly lactate, acetate and ethanol could also represent an ideal feedstock for caproic acid production through chain elongation [57].

In conclusion, the main challenge at this stage is to experimentally demonstrate the selectivity, productivity, and robustness of the mixed culture bioethanol production process. Two enrichment studies have demonstrated a relatively high yield but with low titres using glucose. Opportunities for future mixed culture experimentation lie in increasing the glucose concentration, and thus the ethanol titre. Secondly, the effectiveness of all three described strategies and the synergy of their combination should be assessed. Thirdly, high yield ethanol production needs to be demonstrated using (pre-treated) food waste. In addition to these key experiments, other relevant ecological parameters deserve to be carefully explored, such as fermentation temperature, the protein content or type of fermentable carbohydrate [51**]. We believe that a solid understanding of the ecology of ethanol production will not only be useful for development of mixed culture biotechnology-based processes, but also strengthens pure culture-based fermentation processes, by identifying conditions that intrinsically promote ethanol production.

Conflict of interest statement

Nothing declared.

Acknowledgements

J.L. Rombouts wishes to thank the early support of this work through the SIAM gravitation grant from the Netherlands Organisation for Scientific Research (NWO) under the number 024.002.002. He is also grateful for the discussions with Prof. Dr. G.J. Witkamp on the topic of an integrated bioethanol production processes using microbial communities. He further wishes to thank J.J. de Jongh and B. van de Berg for their effort in co-evaluating the business case of bioethanol and electricity production from food waste (swill) in the Rotterdam area (The Netherlands).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.copbio.2021.01.016>.

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