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REVIEW

Development and application of co-culture for ethanol production by co-fermentation of glucose and xylose: a systematic review

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Abstract This article reviews current co-culture systems for fermenting mixtures of glucose and xylose to ethanol. Thirty-five co-culture systems that ferment either synthetic glucose and xylose mixture or various biomass hydrolysates are examined. Strain combinations, fermentation modes and conditions, and fermentation performance for these co-culture systems are compared and discussed. It is noted that the combination of *Pichia stipitis* with *Saccharomyces cerevisiae* or its respiratory-deficient mutant is most commonly used. One of the best results for fermentation of glucose and xylose mixture is achieved by using co-culture of immobilized *Zymomonas mobilis* and free cells of *P. stipitis*, giving volumetric ethanol production of 1.277 g/l/h and ethanol yield of 0.49–0.50 g/g. The review discloses that, as a strategy for efficient conversion of glucose and xylose, co-culture fermentation for ethanol production from lignocellulosic biomass can increase ethanol yield and production rate, shorten fermentation time, and reduce process costs, and it is a promising technology although immature.

Keywords Ethanol production · Co-culture · Co-fermentation · Glucose · Xylose

Introduction

Ethanol, as a potential alternative to fossil fuels, has attracted a great deal of interest over the past few years. Currently, most ethanol is produced by fermentation of glucose from corn, or sucrose from sugar cane and beets [30, 63, 67, 70, 83, 97]. Competition between fuel and food production, however, is not considered ethical or economically sustainable. Ethanol production from starch or sugar has some negative effects, which come from the fact that ethanol production from starch or sugar cane potentially competes with food production either directly, as in the case of corn, or indirectly by competing with food production for land and water.

Lignocellulosic biomass (such as agricultural residues, forestry wastes, waste paper, municipal solid wastes, and energy crops) has been considered as possible raw material for ethanol production due to its renewability, large quantities, low prices (relative to grain or sugar), and potential environmental benefits [11, 54, 65, 70, 83, 97]. In spite of significant progress in advancing conversion of lignocelluloses to ethanol, one major barrier to achieving widespread commercialization is the lack of cost-competitive processes for ethanol production from mixed sugar hydrolysates [11, 30, 44, 67, 70, 85]. The key requirements for an economical lignocellulosic ethanol process include: (1) efficient pretreatment methods of lignocelluloses, (2) availability of low-cost hydrolytic enzymes, and (3) use of optimal microbial strains capable of converting hexose and pentose sugars to ethanol at high rates, yields, and final concentrations [50, 67, 78, 85].

Glucose and xylose are the two dominant sugars in lignocellulosic hydrolysates. Both need to be fermented efficiently, but current approaches are inefficient, since no native microorganisms can convert all sugars into ethanol at high yield [67, 83]. *Saccharomyces cerevisiae*, which is by far the dominant yeast used for ethanol production, naturally converts glucose to ethanol but does not metabolize xylose [46, 67]. The lack of industrially robust

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microbes for co-fermentation of glucose and xylose has been a major technical barrier.

Researchers have taken two different approaches to solve this problem. One is the construction of genetically modified microorganisms into which pathways for xylose fermentation have been introduced. Some genetically modified microorganisms have been engineered to ferment glucose and xylose at high ethanol yield, such as recombinant *Escherichia coli*, *Klebsiella oxytoca*, *Zymomonas mobilis*, and *S. cerevisiae* [3, 5, 9, 10, 14, 15, 21–24, 37, 39, 41–44, 46, 55, 72, 73, 88, 96, 98–100]. For example, the engineered *E. coli* strain KO11 can produce ethanol from all of the sugars which are constituents of hemicellulose with greater than 95% theoretical yield [44]. The very high yields presumably reflect the use of tryptone and yeast extract present in the medium as adjunct carbon sources. The other approach is to utilize two microorganisms at the same time, which is called "co-culture" (two microorganisms are cultured together and simultaneously exist in the same medium). Utilization of co-cultures for ethanol production appears to have advantages over single culture since there is potential for synergistic action of the metabolic pathways of all involved strains [6]. Multiple microbes are often present during fermentation of traditional beverages or food (such as cheese, yoghurt, sauerkraut, sourdough, salami, beer, whisky, and wine), where they efficiently utilize complex substrates [6, 91].

According to a US Department of Energy (DOE) study, co-culture bioconversion is a very plausible and potentially high-payoff opportunity for ethanol production [20]. Research has been conducted to study utilization of co-culture for ethanol production by co-fermentation of glucose and xylose or various biomass hydrolysates [16–18, 26–28, 31, 32, 34, 36, 38, 51, 52, 57–59, 61, 62, 71, 74, 75, 77, 82, 86, 89–91]. Some research efforts have shown promising results. These include co-culture of immobilized *Z. mobilis* and free cells of *Pichia stipitis* (reclassified as *Scheffersomyces stipitis*) [26], co-culture of ethanologenic *E. coli* strain KO11 with *S. cerevisiae* [75], co-culture of *Z. mobilis* and *Candida tropicalis* for ethanol production from hydrolyzed agricultural wastes [77], co-culture of *S. cerevisiae* and *Pachysolen tannophilis* [74], and co-culture of restricted catabolite repressed mutant *P. stipitis* and respiratory-deficient mutant *S. cerevisiae* [51, 52].

The present review aims to provide an overview of the current state of co-culture systems for ethanol production, discussing the characteristics of different co-culture systems, considering recent advances in this area, pointing out the potential benefits and challenges of using co-culture for co-fermentation of glucose and xylose at industrial scale, and providing valuable information for future research. Some considerations on current and future research trends in co-culture for ethanol production involving selection of combined strains, construction of kinetic models, and metabolic models for stable co-culture systems are also presented.

Current co-culture systems for ethanol production by co-fermentation of glucose and xylose

Co-cultures are industrially applied to wastewater treatment, biogas production, biological soil remediation, and production of traditional foods, such as cheese, yoghurt, pickles, whisky, and so on [6, 91]. Moreover, many environmental bioconversions are catalyzed by mixed microbial cultures in an apparently stable fashion in natural systems. Based on insights into how commercial and natural systems function, the idea of using a co-culture approach for production of ethanol is to combine a xylose-fermenting microorganism and a glucose-fermenting microorganism to ferment glucose and xylose simultaneously.

Interactions between microorganisms in co-culture systems

To have a stable co-culture, certain requirements must exist. One is that the two strains must be compatible and able to grow together. Laplace et al. [56] have studied the compatibility aspects of various strains through Petri plate assay. According to the study, none of the six tested *S. cerevisiae* strains were found to inhibit growth of *P. stipitis* or *C. shehatae*, and none of the five tested *C. shehatae* strains were found to have an inhibitory effect on growth of *Saccharomyces* species. Thus, each strain of *C. shehatae* could be used with *Saccharomyces* species. Among the six tested *P. stipitis* strains, five demonstrated killer activity against *Saccharomyces* species, and three of these five strains showed killer activity against *S. cerevisiae*. Another requirement is that the fermentation conditions, such as pH, temperature, and oxygen supply, for the two strains should be compatible. For example, *Z. mobilis* ferments glucose at pH 7 and temperature of 37°C, but these conditions are not compatible with those of xylose-fermenting yeasts (*P. stipitis* and *C. shehatae*), which need pH 5 and temperature 30°C. In contrast, the pH and temperature at which *S. cerevisiae* ferments glucose to ethanol are compatible with those of xylose-fermenting yeasts. Therefore, a combination of *P. stipitis* or *C. shehatae* and *S. cerevisiae* could and has been used in co-culture processes.

Compared with pure culture, interactions between the different microorganisms play a critical role in co-culture systems. The interactions can occur either through direct cell-to-cell communications or by signal substances in the fermentation broth [6]. Specifically, stable co-culture could be controlled by metabolic interactions (i.e., syntrophic

relationships, or competition for substrates) and other interactions (i.e., growth promoters or inhibitors such as antibiotics) [68]. The possible interactions between two microbial species can be categorized as positive, negative, and neutral [82]. For example, positive interaction in co-cultures may take place through reduction of available oxygen by aerobic microbes, creating anaerobic conditions that promote growth of anaerobic or microaerophilic strains. This kind of microbial mixed culture provides protection from environmental influences. Negative interaction could happen when two microorganisms compete for the same resource, such as space or a limiting nutrient. Neutral interaction means that there is no effect when the two populations are present together. For a desirable co-culture system, positive interactions between the two microorganisms are expected. However, the interactions between microorganisms in mixed culture environments may not always lead to desirable consequences. Therefore, understanding the interactions between associated strains in a co-culture system is very important. However, very little research has been done so far, primarily due to the complex nature of systems containing multiple microorganisms. This offers new avenues for future research.

Strains used in current co-culture systems

Table 1 presents an overview of co-culture systems for ethanol production that have appeared in the literature since 1981. In this section, we will discuss the combinations of different microorganisms and review the methods and principles that can be used to screen strains capable of co-fermentation of glucose and xylose.

The principal naturally ethanologenic microorganisms include a number of yeasts and bacteria. Yeasts include *S. cerevisiae*, *Kluyveromyces marxianus*, *P. stipitis*, *C. shehatae*, *P. tannophilius*, and so on. Bacteria that can produce ethanol from biomass include *E. coli*, *Z. mobilis*, *Zymobacter palmae*, *Clostridium cellulolyticum*, *Clostridium thermocellum*, *Clostridium thermosaccharolyticum* (now classified as *Thermoanaerobacterium thermosaccharolyticum*), and *Bacillus stearothermophilus*. Among these microorganisms, *S. cerevisiae* is normally used for ethanol fermentation, but most wild strains of *S. cerevisiae* are not able to metabolize xylose; *P. stipitis*, *C. shehatae*, and *P. tannophilius* are found to be capable of fermenting xylose [29, 51]. When selecting combined microbial species for a co-culture system, the first step is to choose a glucose-fermenting microorganism and a xylose-fermenting microorganism, then test their compatibility and study their co-fermentation performance.

From Table 1, it can be seen that the yeast genus *Saccharomyces* is preferably used as the glucose-fermenting strain with a xylose-fermenting strain. Many researchers prefer to use *P. stipitis* as the xylose-fermenting strain with a glucose-fermenting strain in their co-culture systems. The most commonly used strain combination is *P. stipitis* and *S. cerevisiae* or its respiratory-deficient mutant. The reason for this preference is that the pH and temperature at which *S. cerevisiae* ferments glucose to ethanol are compatible with those of *P. stipitis*. Low levels of oxygen (approximately 2 mmol $l\ h^{-1}$) are necessary for efficient ethanol formation from xylose by the xylose-fermenting yeasts in order to maintain cell viability and nicotinamide adenine dinucleotide (NADH) balance [12, 31, 32, 34]. However, *S. cerevisiae* does not require oxygen to ferment glucose. Respiratory-deficient mutant strains of *S. cerevisiae* have been utilized in co-culture systems to solve this conflict problem of oxygen supply [17, 35, 36, 57, 58, 89], since a respiratory-deficient *Saccharomyces* mutant can generate an oxygen profile favorable to xylose-fermenting yeast [17, 18]. The other issue with using the combination of *P. stipitis* and *S. cerevisiae* is that rapid formation of ethanol from glucose in the co-culture scheme may induce inhibition of xylose fermentation due to the low ethanol tolerance of *P. stipitis* [12]. Delgenes et al. applied continuous culture conditions to address this problem [17, 18]. Under continuous fermentation, glucose concentration can be kept sufficiently low so as not to repress xylose utilization by the xylose-fermenting yeast. Apart from *S. cerevisiae*, *S. diastaticus*, *Z. mobilis*, *K. marxianus*, and *C. thermocellum* have served as the glucose-fermenting microorganism in some co-culture systems [18, 26, 38, 57, 59, 71, 81]. On the other hand, *P. tannophilus*, *C. tropicalis*, *C. shehatae*, *K. fragilis*, and recombinant *E. coli* have been used in co-cultures systems as the xylose-fermenting microorganism in place of *P. stipitis* [7, 27, 62, 74, 75, 77, 88].

Fermentation modes

Three fermentation modes can be used in co-culture systems: batch, continuous, and fed-batch. Abbi et al. conducted fermentation of xylose and rice straw hydrolysate by *C. shehatae* in batch, fed-batch, and continuous culture conditions and found that fed-batch or continuous cultures exhibited higher ethanol yields and volumetric productivities [2]. For co-culture systems, the selection of fermentation mode depends on the microbes in the system. Laplace et al. compared batch and continuous fermentation of glucose/xylose mixture by a respiratory-deficient mutant of *S. cerevisiae* co-cultivated with *C. shehatae* [58]. Their results showed that xylose was poorly utilized in batch condition (only 6%), but continuous condition provided simultaneous conversion of glucose and xylose, because the high fermentative potential of *S. cerevisiae* generated glucose concentrations low enough to allow xylose conversion.

Table 1 Summary of different co-culture systems

Co-culture systems	Fermentation mode	Feedstock/medium	Fermentation condition	Performance	Reference
Clostridium thermocellum CT2–*Clostridium thermosaccharolyticum* HG8	Batch	Alkali treated banana waste (100 g/l)	Inoculum size: 5% (v/v) Temperature: 60°C pH: 7.5 Fermentation time: 5 days	$Y_{p/s}$: 0.41 $C_{E,max}$: 22	Harish Kumar Reddy et al. [38]
Z. mobilis–*P. stipitis*	Batch	Glucose/xylose sugar mixture (30 g/l glucose and 20 g/l xylose)	The co-culture includes immobilized *Z. mobilis* in the calcium alginate gel beads and free *P. stipitis* Temperature: 30°C Stirring speed: 150 rpm Working volume: 800 ml Air flow rate level: 80 cm^3/min	$Y_{p/s}$: 0.49–0.50 Q_P: 1.277	Fu et al. [26]
E. coli KO11–*S. cerevisiae* TJ1	Batch	Waste house wood hydrolysate medium (27.0 g/l glucose and 17.0 g/l xylose) containing 1% (v/v) corn steep liquor	Inoculum size: 0.2 g-dry cell weight/l *E. coli* and 0.02 g-dry cell weight/l *S. cerevisiae* Temperature: 35°C pH: 6.0 (controlled by 10 N KOH) Working volume: 200 ml Shaking rate: 80 rpm The oxygen transfer rate: 5–7 mmol/(l h)	S_K: 46 $Y_{p/s}$: 0.43 $C_{E,max}$: 30.3	Okuda et al. [75]
Z. mobilis–*P. tannophilus* (successive inoculation)	Batch	Glucose/xylose mixture (60 g/l glucose and 40 g/l xylose)	Temperature: 30°C Working volume: 900 ml Inoculations were carried out successively with firstly *Z. mobilis*, and after all the glucose had been converted to ethanol. *P. tannophilus* was then inoculated. Cofermentation with no aeration at glucose fermentation stage and a aeration level <1 mmol/l/h at xylose fermentation stage	$Y_{p/s}$: 0.33 X_{max}: 5.1×10^7 (*Z. mobilis*) X_{max}: 5.7×10^7 (*P. tannophilus*) Q_P: 2.32	Fu and Peiris [27]
Z. mobilis MTCC 92–*C. tropicalis* TERI SH110	Batch	Hydrolyzed fruit and vegetable residues	Inoculum size: 10% (v/v) Working volume: 50 ml Temperature: 30°C	Mixed culture can yield 97.7% of the theoretical yield of ethanol from enzymatic hydrolysis	Patle and Lal [77]
P. stipitis CCUG18492–*K. marxianus*	Batch	Sugar mixture (30 g/l glucose, 30 g/l xylose, 12 g/l mannose, 8 g/l galactose)	pH: 4.5 Working volume:100 ml Shaking rate:100 rpm	$Y_{p/s}$: 0.36 $Y_{x/s}$: 0.08 Q_{pmax}: 1.08 $C_{E,max}$: 31.87 E: 99	Rouhollah et al. [81]

Table 1 continued

Co-culture systems	Fermentation mode	Feedstock/medium	Fermentation condition	Performance	Reference
P. stipitis CCUG18492–*S. cerevisiae*	Batch	Sugar mixture (30 g/l glucose, 30 g/l xylose, 12 g/l mannose, 8 g/l galactose)	pH: 4.5 Working volume: 100 ml Shaking rate: 100 rpm	$Y_{p/s}$: 0.41 $Y_{x/s}$: 0.08 Q_{pmax}: 0.77 $C_{E,max}$: 29.45 E: 94	Rouhollah et al. [81]
S. cerevisiae 2.535–*P. tannophilis* ATCC 2.1662	Batch	Treated or untreated softwood hydrolysate	Working volume: 150 ml hydrolysate and 50 ml inoculum of co-cultures Temperature: 30°C pH: 5.5	For treated softwood hydrolysate fermentation by adapted co-culture $Y_{p/s}$: 0.49 Q_P: 0.38 Sugar consumed (%): >99 Ethanol (g/L): 18.2	Qian et al. [74]
S. cerevisiae–recombinant *E. coli*	Batch	Treated or untreated softwood hydrolysate	Working volume: 150 ml hydrolysate and 50 ml inoculum of co-cultures Temperature: 30°C pH: 7.0	For treated softwood hydrolysate fermentation by adapted co-culture $Y_{p/s}$: 0.45 Q_P: 0.71 Sugar consumed (%): >99 Ethanol (g/l): 17.1	Qian et al. [74]
P. stipitis NRRL Y-11544)–*S. cerevisiae* (Baker Yeast Type II) coimmobilized in Ca-alginate gel beads	Batch	Mix sugar syrups (45 g/l glucose and 12 g/l xylose) and enzymatic hydrolysates of stream-exploded aspen chips (40 g/l glucose and 10 g/l xylose)	Temperature: 30°C pH: 5.5	Best conditions for the co-fermentation of 40 g/l glucose and 10 g/l xylose $Y_{p/s}$: 0.396 Conversion: 0.995 g consumed/g initial	De Bari et al. [16]
Z. mobilis 3881–*K. fragilis*	Batch	Hydrolyzed mashed tubers	Working volume: 200 g medium and 20 ml inoculums Temperature: 30°C	E: 99 $Y_{p/s}$: 0.48	Szambelan et al. [86]
Restricted catabolite repressed mutant *P. stipitis* CCY39501(P5-200-16)–respiratory deficient mutant *S. cerevisiae* Ja(a)	Batch	Glucose/xylose mixture (35 g/l glucose and 15 g/l xylose)	Temperature: 28°C pH: 5.5 Fermentation time: 120 h 1% v/v of respiratory deficient mutant *S. cerevisiae* V_{30} and 1% v/v of *P. stipitis* were inoculated in 150 ml F_3 medium	S_G: 100.00 S_K: 68.00 $C_{E,max}$: 20.30 $Y_{p/s}$: 0.45 $Y_{x/s}$: 0.20 Q_P: 0.169	Kordowska-wiater and Targoński [52]

Table 1 continued

Co-culture systems	Fermentation mode	Feedstock/medium	Fermentation condition	Performance	Reference
S. cerevisiae–C. tropicalis	Batch	Alkali hydrolyzed corn cobs	Temperature: 37°C Shaking rate: 150 rpm	$C_{E,max}$: 27 Q_P: 0.28	Latif and Rajoka [61]
P. stipitis CCY39501–*S. cerevisiae* V_{30}	Batch	Glucose/xylose mixture (35 g/l glucose and 15 g/l xylose)	Temperature: 28°C pH: 5.5 (controlled by 15% NaOH) Fermentation time: 96 h 2% v/v of respiratory deficient mutant *S. cerevisiae* V_{30} and 1% v/v of *P. stipitis* were inoculated in 150 ml medium	S_G: 99.71 S_K: 26.67 $C_{E,max}$: 15.00 $Y_{p/s}$: 0.39 $Y_{x/s}$: 0.14 Q_P: 0.318	Kordowska-Wiater and Targoński [51]
P. stipitis CCY39501–respiratory deficient mutant *S. cerevisiae* V_{30}	Batch	Glucose/xylose mixture (35 g/l glucose and 15 g/l xylose)	Temperature: 28°C pH: 5.5 (controlled by 15% NaOH) Fermentation time: 96 h 2% v/v of respiratory deficient mutant *S. cerevisiae* V_{30} and 1% v/v of *P. stipitis* were inoculated in 150 ml medium	S_G: 100.00 S_K: 99.67 $C_{E,max}$: 18.80 $Y_{p/s}$: 0.38 $Y_{x/s}$: 0.12 Q_P: 0.264	Kordowska-Wiater and Targoński [51]
P. stipitis NRRL 11545–respiratory deficient mutant of *S. cerevisiae* CBS 1200	Continuous	Glucose/xylose mixture (35 g/l glucose and 15 g/l xylose)	Inoculum size: 3% (v/v) (corresponding to an initial dry cell concentration of 0.2 g/l) Working volume:1.5 l $D = 0.1\ h^{-1}$	S_G: 100 S_K: 60 E: 88 $C_{E,max}$: 19 $Y_{p/s}$: 0.43 Q_P: 2	Delgenes et al. [17]
S. cerevisae CBS 1200–*C. shehatae* ATCC 22984 coimmobilized in a two-chambered bioreactor	Batch	Glucose/xylose mixture (35 g/l glucose and 15 g/l xylose)	Initial cell loading: *C. shehatae* 0.65 mg dry wt/ml *S. cerevisiae* 5.00 mg dry wt/ml Temperature:30 pH: 5.0 (phosphate buffer) magnetic stirring	Best conditions: $Y_{p/s}$: 0.47 $Y_{x/s}$: 0.088 Q_P: 7.5	Lebeau et al. [62]
P. stipitis CBS5773–*S. cerevisiae* no. 7	Batch	Glucose/xylose mixture (50 g/l glucose and 25 g/l xylose)	The initial concentration of *P. stipitis* and *S. cerevisiae* were 7.1 g/l and 1.5 g/l, respectively. pH: 5.0 (controlled by 2 N NaOH and 2 N HCl) Working volume: 1 l Fermentation time: 40 h qO_2 was controlled at 66.7 mg/g cell/h for glucose consumption and then controlled at 14.3 mg/g cell/h for xylose consumption	$Y_{p/s}$: 0.39 $C_{E,max}$: 29.4 Q_P: 0.74	Taniguchi et al. [89]

Table 1 continued

Co-culture systems	Fermentation mode	Feedstock/medium	Fermentation condition	Performance	Reference
P. stipitis CBS5773–respiratory deficient mutant *S. cerevisiae* no. 7	Batch	Glucose/xylose mixture (50 g/l glucose and 25 g/l xylose)	The initial concentration of *P. stipitis* and *S. cerevisiae* were 7.1 g/l and 1.5 g/l, respectively. pH: 5.0 (controlled by 2 N NaOH and 2 N HCl) Working volume: 1 l Fermentation time: 40 h	$Y_{p/s}$: 0.50 $C_{E,max}$: 37.5 Q_P: 0.94	Taniguchi et al. [89]
P. stipitis CBS5773–*S. cerevisiae* no. 7	Batch	Glucose/xylose mixture (50 g/l glucose and 25 g/l xylose)	The initial concentration of *P. stipitis* and *S. cerevisiae* were 3.5 g/l and 0.75 g/l, respectively. Fermentation time: 68 h qO_2 was controlled at 66.7 mg/g cell/h for glucose fermentation stage and 14.3 mg/g cell/h for xylose fermentation stage	$Y_{p/s}$: 0.35 $C_{E,max}$: 26.2 Q_P: 0.39	Taniguchi et al. [90]
P. stipitis CBS5773 (fermentor A)–*S. cerevisiae* no. 7 (fermentor B)	Batch	Glucose/xylose mixture (50 g/l glucose and 25 g/l xylose)	The initial concentration of *P. stipitis* and *S. cerevisiae* were 7.0 g/l and 0.75 g/l, respectively. Filtered air was sparged at constant flow rate (0.2 vvm) into fermentor A. Meanwhile, nitrogen gas was sparged at 0.2 vvm into fermentor B. Fermentation time: 56 h Working volume in fermentor A: 1 l Working volume in fermentor B: 1 l Total working volume:2 l	For *P. stipitis* in fermentor A $Y_{p/s}$: 0.44 $C_{E,max}$: 33.1 Q_P: 0.59 For *S. cerevisiae* in fermentor B $Y_{p/s}$: 0.45 $C_{E,max}$: 33.7 Q_P: 0.60	Taniguchi et al. [90] Taniguchi and Tanaka [91]
P. stipitis NRRL Y7124–respiratory deficient mutant of *S. diastaticus NCYC 625*	Continuous	Complete hydrolysis of aspen wood (41.1 g/l glucose and 9 g/l xylose)	Volume:1.5 l pH: 5.0 Temperature: 30 °C The fermentor was inoculated at 3% v/v, corresponding to an initial dry cell concentration of 0.2 g/l. The dilution rate was increased by stages from 0.038 h^{-1} to 0.125 h^{-1}	E:100 $C_{E,max}$: 13.5 $Y_{p/s}$: 0.25 Q_P: 1.6 (Fermentation performance were given at D = 0.125 h^{-1})	Delgenes et al. [18]
Z. mobilis–Saccharomyces sp.	Batch	Sucrose-based medium (200 g/l reducing sugars)	20 ml mixed culture containing 10 ml *Z. mobilis* inoculum and 10 ml *Saccharomyces* sp. inoculum was used. Working volume: 0.5 l	$Y_{p/s}$: 0.5 Q_P: 1.5	Abate et al. [1]

Table 1 continued

Co-culture systems	Fermentation mode	Feedstock/medium	Fermentation condition	Performance	Reference
P. stipitis–respiratory deficient mutant *S. diastaticus*	Continuous	Glucose/xylose mixture (70% glucose and 30% xylose) Different substrate concentrations (20 g/l, 50 g/l, 80 g/l) were tested at $D = 0.015\ h^{-1}$	Inoculum size: 3% (v/v) Temperature: 30°C pH: 5.0 Working volume: 1.5 l Stirring speed: 800 rpm O_2 transfer rate: 1.75 mmol/l/h	Influence of dilution rate and influence of initial sugar concentration on the fermentation performance were studied. An average ethanol concentration of 18.6 g/l was obtained with $D = 0.023\ h^{-1}$ and initial substrate concentration of 50 g/l. The maximum ethanol concentration of 27.5 g/l was reached with initial substrate concentration of 80 g/l and dilution rate of $0.015\ h^{-1}$, giving an ethanol yield of 0.45 g/g	Laplace et al. [57]
C. shehatae ATCC 22984–*S. cerevisiae* CBS 1200	Batch	Glucose/xylose mixture (14 g/l glucose and 6 g/l xylose)	Inoculums of each yeast strain were 1.5%. Temperature: 30°C pH: 5.0 Working volume: 1.5 l Stirring speed: 800 rpm Aeration rate: 0.005 vvw Oxygen transfer rate: 1.75 mmol/l/h	$C_{E,max}$: 14.5 $Y_{p/s}$: 0.39 S_G: 100 S_K: 8	Laplace et al. [58]
C. shehatae ATCC 22984–respiratory deficient mutant *S. cerevisiae* CBS1200	Batch	Glucose/xylose mixture (14 g/l glucose and 6 g/l xylose)	Inoculums of each yeast strain were 1.5%. Temperature: 30°C pH: 5.0 Working volume: 1.5 l Stirring speed: 800 rpm Aeration rate: 0.005 vvw Oxygen transfer rate: 1.75 mmol/l/h	$C_{E,max}$: 14.7 $Y_{p/s}$: 0.40 S_G: 100 S_K: 6	Laplace et al. [58]
C. shehatae ATCC 22984–respiratory deficient mutant *S. cerevisiae* CBS1200	Continuous	Glucose/xylose mixture (14 g/l glucose and 6 g/l xylose)	Inoculums of each yeast strain were 1.5%. Temperature: 30°C pH: 5.0 Working volume: 1.5 l Stirring speed: 800 rpm Aeration rate: 0.005 vvw Oxygen transfer rate: 1.75 mmol/l/h	$C_{E,max}$: 14.5 $Y_{p/s}$: 0.39 S_G: 100 S_K: 8	Laplace et al. [58]

Table 1 continued

Co-culture systems	Fermentation mode	Feedstock/medium	Fermentation condition	Performance	Reference
P. stipitis–respiratory deficient mutant *S. diastaticus*	Continuous	Glucose/xylose mixture (35 g/l glucose and 15 g/l xylose)	Inoculum size: 3% (v/v) Temperature: 30°C pH: 5.0 Filtered air was supplied at the bottom of the fermenter through a capillary tube to generate agitation and through a fritted tube to provide the oxygen supply for xylose fermentation	E: 94 $C_{E,max}$: 21.5 $Y_{p/s}$: 0.45 Q_P:4.3 (observed at $D = 0.20\ h^{-1}$)	Laplace et al. [59]
Coimmobilized *P. stipitis* CBS 5773–*S. cerevisiae* CBS 8066	Continuous	Glucose/xylose mixture	Temperature: 30°C pH: 5.0 Working volume: 400 ml	Best condition: D: 0.07 Influent concentration: Glucose: 40.9 g/l Xylose: 10.5 g/l Effluent concentration: Glucose: 0.02 g/l Xylose: 6.6 g/l	Grootjen et al. [33]
*I*mmobilized *P. stipitis* CBS 5773–suspended *S. cerevisiae* CBS 8066	Continuous	Glucose/xylose mixture	Temperature: 30°C pH: 5.0 Working volume: 400 ml	Best condition: D: 0.11 Influent concentration: Glucose: 40.5 g/l Xylose: 11.2 g/l Effluent concentration: Glucose:0.0 g/l Xylose: 10.0 g/l	Grootjen et al. [33]
Coimmobilized *P. stipitis* CBS 5773–*S. cerevisiae* CBS 8066	Continuous	Glucose/xylose mixture (40 g/l glucose and 10 g/l xylose)	Temperature: 30°C pH: 5.0 Working volume: 200 ml	Best condition: D: 0.375 $Y_{p/s}$: 0.4 $C_{E,max}$: 20	Grootjen et al. [31]
P. tannophilus m–S. cerevisiae	Batch	Mixed sugar fermentation media (46% glucose, 44% xylose, 4% mannose, 2% arabinose, 4% galactose)	Temperature: 30°C Working volume: 100 ml Shaking rate:100 rpm	$Y_{p/s}$: 0.46 $C_{E,max}$: 27 (Result was at the initial sugar concentration of 125 g/l)	Beck et al. [7]
P. tannophilus s–S. cerevisiae	Batch	Mixed sugar fermentation media (46% glucose, 44% xylose, 4% mannose, 2% arabinose, 4% galactose)	Temperature: 30°C Working volume: 100 ml Shaking rate:100 rpm	$Y_{p/s}$: 0.44 $C_{E,max}$: 42.7 (Result was at the initial sugar concentration of 200 g/l)	Beck et al. [7]

Table 1 continued

Co-culture systems	Fermentation mode	Feedstock/medium	Fermentation condition	Performance	Reference
E. coli–S. cerevisiae	Batch	Mixed sugar fermentation media (46% glucose, 44% xylose, 4% mannose, 2% arabinose, 4% galactose)	Temperature: 30°C Working volume: 100 ml Shaking rate: 100 rpm	$Y_{p/s}$: 0.43 $C_{E,max}$: 41.2 (Result was at the initial sugar concentration of 200 g/l)	Beck et al. [7]
C. thermocellum LQRI–*C. thermohydrosulfuricum* 39E	Continuous	Various saccharides derived from cellulosic biomass (MN300 cellulose, Avicel, Solka Floc, SO_2-treated wood, and steam-exploded wood) were examined	The inoculums size was 1.5% or 1.0×10^7 to 5×10^7 cells of each species per ml (final concentration). Temperature: 60°C pH: 7.0 Agitation speed: 100 rpm	The highest ethanol yield obtained was 1.8 mol of ethanol per mol of anhydroglucose unit in MN300 cellulose.	Ng et al. [71]

$Y_{p/s}$ overall Ethanol yield (g ethanol/g substrate), X_{max} maximum biomass yield (CFU/ml), Q_P volumetric productivity of ethanol (g/l/h), Q_{pmax} maximum volumetric ethanol productivity (g/l/h), $C_{E,max}$ maximum ethanol concentration (g/l), $Y_{x/s}$ cell biomass yield (g cell biomass/g substrate), E efficiency of substrate utilization (%), S_G efficiency of glucose utilization (%), S_K efficiency of xylose utilization (%), qO_2 specific oxygen uptake rate, mg/g cell/h, D diluting rate (h^{-1})

As shown in Table 1, most current co-culture systems were operated in batch mode, and some co-cultures were operated in continuous mode, but no systems used a fed-batch approach. Although batch mode is simple and easily controlled, it has some limitations. One is that the glucose can suppress xylose fermentation, especially at the initial stage, because xylose conversion is completely inhibited at glucose concentration of 2.3 g/l and higher [33]. In a continuous fermentation co-culture system, glucose concentration can be kept sufficiently low so as not to repress xylose utilization by the xylose-fermenting yeast [17, 18, 33]. For example, control can be obtained by adjusting the dilution rate so the glucose concentration can be kept below 2.3 g/l; hence, fast and simultaneous conversion of glucose and xylose in co-cultured systems can be easily achieved by continuous fermentation. Another way to achieve low glucose concentration is by use of *S. cerevisiae*; its high fermentative potential allows fast xylose conversion by generating a low glucose concentration environment [58]. This approach, however, can be limited if the amount of ethanol produced from glucose exceeds the ethanol tolerance of the xylose-fermenting organism. When the glucose is close to being depleted, the high ethanol concentration (around 30 g/l) can inhibit the xylose fermentation process [81]. Continuous fermentation with medium outflow can avoid accumulation of ethanol and other inhibiting metabolites in the system [62]. This, however, is potentially subject to instabilities if the two organisms show biphasic growth or very different growth rates. Wash out is one of the critical issues for this mode, even with a very low dilution rate [87]. Different methods for cell retention (immobilization, encapsulation, filtration) and for cell recirculation (using centrifuges or flocculating organisms) can be used to overcome this problem. Grootjen et al. co-immobilized cells of *S. cerevisiae* and *P. stipitis* in alginate beads in order to continuously ferment a synthetic glucose and xylose mixture [31, 34]. Filtration can be applied to keep cells inside a chemostat when a membrane is added at the outlet. Instead of using a cell retention method, Abbi et al. aseptically removed cells by centrifugation after 24 h of fermentation, then transferred to a fresh medium and incubated further [2].

So far, fed-batch mode has not been utilized in any co-culture system. However, this mode can affect culture growth and avoid overflow metabolism (such as acetate for *E. coli*) due to the feeding of a growth-limiting nutrient substrate. In addition, fed-batch mode can solve the problem that arises when the concentration of inhibitors begins to impact on ethanol production for pentose-fermenting yeasts by maintaining an optimum dilution rate [11]. Therefore, it has advantages for use in co-culture systems.

Fermentation conditions

The fermentation conditions for a co-culture system depend mainly on the selection of the two microorganisms. Each selected pair of microorganisms will have its own optimum values for temperature, pH, aerobic or anaerobic environment, and inoculum size.

As shown in Table 1, all of the co-culture fermentations tried thus far have been conducted at laboratory scale with working volume of 1.5 l or less. For simplicity, most researchers use a synthetic medium (a mixture of glucose and xylose). For co-fermentation of glucose and xylose, both the initial total sugar concentration and the proportion of glucose and xylose play important roles in affecting fermentation performance. However, very limited studies have been performed to understand exactly how these two factors interact in co-culture systems. The only known research on the effects of initial total sugar concentration is by Laplace et al. [57], but this effort did not address the question of how different sugar compositions could affect the system. In addition, Laplace et al. investigated the effects of initial total sugar concentration on the fermentative performance of pure cultures for *P. stipitis*, *C. shehatae*, *S. cerevisiae*, and *Z. mobilis* [60]. Reviewing the experimental details of current co-culture systems did not reveal any insights into how the initial sugar concentrations were created. The initial total sugar concentration is selected between 20 and 100 g/l. Generally, the xylose composition varied from 20% to 50%, and most researches used 30%. This value is close to the xylose composition in corn stover hydrolysate and other biomass hydrolysates. This is a reasonable approach, since biomass hydrolysates such as hydrolyzed fruit and vegetable residues [77], banana agrowaste hydrolysate [38], Jerusalem artichoke [86], waste house wood hydrolysate [75], and dry corn cobs [61] have been used in co-culture systems to produce ethanol. Such work has shown that co-culture systems hold great promise for ethanol production from biomass.

Most current co-culture systems used fermentation temperature of 30°C (Table 1). This is especially true for co-culture systems that use the combination of *P. stipitis* and *S. cerevisiae*, since 30°C is optimum for *S. cerevisiae* to ferment glucose and for *P. stipitis* to ferment xylose. The optimal fermentation temperature for *C. thermocellum* and *C. thermosaccharolyticum* is approximately 60°C [38, 63, 68, 71]. Harish Kumar et al. [38] have successfully applied this combination for conversion of banana waste to ethanol with maximum ethanol yield of 0.41 g/g. However, the increased production of byproducts such as acetate and lactate decrease ethanol production by slowing the growth rate of cells [68]. The co-culture system with the combination of *C. thermocellum* and *C. thermohydrosulfuricum* also needed 60°C due to the application of thermophilic anaerobic species. Ng et al. [71] created a stable co-culture that contained nearly equal numbers of *C. thermocellum* and *C. thermohydrosulfuricum* for fermenting various cellulosic substrates, and the ethanol yield was twofold higher than in *C. thermocellum* monoculture fermentations. For the combination of *Z. mobilis* and *P. stipitis*, despite the conflict of optimal temperatures for the two stains, researchers still selected 30°C as the fermentation temperature. One possible reason for this is that the co-culture system can be kept stable around 30°C.

Since most researchers are familiar with the combination of *S. cerevisiae* and *P. stipitis*, and pH 5.0 is the optimal value for fermentation by these two yeast strains, this value was used in most co-cultures systems. Usually, the pH of the fermentation medium is controlled at 4.5–7.5 by adding sodium/potassium hydrate or hydrogen chloride. For the combination of *E. coli* and *S. cerevisiae*, Okuda et al. [74] controlled the pH at 6.0 by adding 10 N KOH, but Qian et al. [75] kept the system at pH 7.0. This difference is probably attributable to differences in the hydrolysates. Okuda et al. used waste house wood hydrolysate containing 1% v/v corn steep liquor in their experiments, while Qian et al. used treated or untreated softwood hydrolysate as fermentation medium. With regard to the combinations of two thermophilic anaerobic species, one system controlled pH at 7.0 [71] and the other system selected pH 7.5 [38].

In addition to the effects of fermentation medium, temperature, and pH, oxygen is an important parameter for some co-culture systems, such as those using *P. stipitis* for xylose fermentation. Fermentation and growth of these yeasts occur simultaneously in a low-oxygen environment [63]. For example, *P. stipitis* induces fermentative activity in response to oxygen limitation [47]. Skoog and Hahn-Hägerdal [84] found in their work on xylose fermentation by *P. stipitis* CBS6054 that maximum specific productivity and ethanol yield were achieved when the oxygen transfer rate (OTR) dropped below 1 mmol $l\ h^{-1}$. Some researchers took steps to control the amount of oxygen in the environment by changing the air flow and/or agitation rate in co-culture systems [57, 58, 89]. Taniguchi et al. [89] controlled the average specific oxygen uptake rate at 66.7 mg/g cell/h for glucose consumption and then controlled at 14.3 mg/g cell/h for xylose consumption in the co-culture of *P. stipitis* and respiratory-deficient mutant *S. cerevisiae*. Laplace et al. maintained the OTR at 1.75 mmol $l\ h^{-1}$ in their co-culture systems [57, 58]. To solve the problem of the different oxygen requirements of *Z. mobilis* and *P. stipitis*, Fu et al. [26, 27] used sequential culture or a modified fermentor by adding a sieve plate and a moving device to a normal fermentor. For co-culture of *E. coli* and *S. cerevisiae*, Okuda et al. controlled the OTR at 5–7 mmol $l\ h^{-1}$ by using the sodium sulfite method [75].

The final parameter for consideration is inoculum size. However, there is little information published on how relative inocula volumes influence fermentation performance. As indicated in Table 1, the inoculum volume in current co-culture systems ranged from 2% to 10% (v/v), and most co-culture systems selected inocula size of 3% (v/v). As to the ratio of the glucose-fermenting microorganism to the xylose-fermenting microorganism, Laplace et al. [57–59], Ng et al. [71], and Kordowska-Wiater and Targoński [52] chose to use the same amount of the two species in their systems. However, Lebeau et al. [62] and Okuda et al. [75] used different amounts (ratios of about 7.69:1 and 1:10, respectively), due to differences in the fermentation medium composition.

Fermentation performance

Several parameters such as ethanol yield ($Y_{p/s}$), volumetric ethanol productivity (Q_p), specific ethanol production rate (q_p), efficiency of substrate utilization (E) or other measures are used to evaluate fermentation processes. Among these, ethanol yield (g ethanol/g consumed sugar substrate) is most useful. The theoretical ethanol yield for glucose/xylose fermentation is 0.51 g/g [53]. However, neither sugar yields this much ethanol in practice. With respect to pure cultures, higher ethanol yields have been achieved by using recombinant *E. coli* KO11 and *Z. mobilis* ZM4 than with recombinant *Saccharomyces* strain 1400 [23]. The recombinant strain *E. coli* KO11 can achieve yield of 0.48 and 0.52 g/g for glucose and xylose, respectively; this represents 94% of the theoretical yield for glucose, and more than 100% for xylose [3]. Alterthum and Ingram hypothesized that the higher than theoretical yield on xylose resulted from the use of tryptone and yeast extract present in the medium as adjunct carbon sources [3]. When using the recombinant strain *Z. mobilis* ZM4 (pZB5), sugar concentrations of approx 50 g/l glucose and 50 g/l xylose can result in ethanol productivity of approximately 5 g/l/h, and yield of 0.50 g ethanol/g substrate, which is 98% of the theoretical yield [49].

From Table 1 it can be seen that the overall ethanol yield of different co-culture systems ranged from 0.25 to 0.50 g/g. The highest overall ethanol yield was obtained at 0.49–0.5 g/g, which is about 98% of the theoretical yield [26, 89]. By employing the strain combination of *P. stipitis* and respiratory-deficient mutant *S. cerevisiae* in batch fermentation, Taniguchi et al. [89] successfully achieved complete sugar utilization in 40 h at volumetric ethanol productivity of 0.94 g/l/h and ethanol yield of 0.50 g/g. More recently, co-culture of immobilized *Z. mobilis* and free cells of *P. stipitis* on a mixture of 30 g/l glucose and 20 g/l xylose resulted in complete conversion to ethanol within 19 h, giving higher volumetric productivity of 1.277 g/l/h and high ethanol yield of 0.49–0.50 g/g [26]. Extension of this fermentation scheme to sugarcane bagasse hydrolysate resulted in complete sugar utilization within 26 h with ethanol yield of 0.49 g/g [26]. These promising results show that co-culture has great potential for efficient conversion of lignocellulosic biomass to ethanol.

Potential benefits and challenges to using co-culture for co-fermentation of hexose and pentose at industrial scale

As discussed in the previous section, all the co-culture systems for co-fermentation of glucose and xylose cited in this paper have been performed at laboratory scale. Use of co-culture for ethanol production has not found wide application at industrial scale, because there are still some serious challenges.

One of the major challenges to the co-culture process is the apparently low ethanol tolerance of xylose-fermenting yeasts [17, 59, 60, 62]. Delgenes et al. [19] reported that ethanol inhibition of *P. stipitis* occurs at ethanol concentration of 30 g/l. The rapid formation of ethanol from glucose in the co-culture system raises the possibility of inhibition of xylose fermentation by ethanol. Improvement of glucose and xylose co-fermentation may depend upon decreasing the influence of ethanol through selection of more ethanol-tolerant strains or use of ethanol removal systems coupled to the fermentation [17].

Another major challenge is finding optimal operating ranges for process parameters (pH, temperature, and oxygen demand) and the acceptable ranges of substrate that can enable optimal activity of each strain in co-culture. Unlike pure culture, co-culture organisms can differ with respect to pH, temperature, and oxygen requirements. Therefore, compromises in process parameters are sometimes necessary; for example, competition for oxygen resulted in low xylose conversion by co-immobilized *S. cerevisiae* and *P. stipitis* [31, 34]. By contrast, using a respiratory-deficient *Saccharomyces* mutant can provide an oxygen environment favorable to xylose-fermenting yeast [17, 18, 51, 52, 57–59, 89]. Improvements in technology for immobilizing cells and innovative fermentor designs could help to solve this problem [26, 90].

Lignocellulosic hydrolysates contain a broad range of inhibitory and toxic compounds, the composition and concentration of which depend upon the type of lignocellulosic materials and the pretreatment and hydrolysis processes. These inhibitory and toxic compounds have a significant and negative impact on pure culture fermentation, and even co-culture fermentation. Due to use of synthetic medium, most laboratory co-cultures did not

encounter these problems. One approach for solving this problem is to remove the inhibitory and toxic compounds or apply detoxification by liming, steam stripping or other methods before the fermentation process starts. Unfortunately, this approach adds considerably to the operational costs. Another approach is adaptation, which has been shown to be an efficient method for increasing tolerance to inhibitors of a broad range of yeast strains in lignocellulosic hydrolysates [10, 89]. Amartey and Jeffries [4] showed that adaptation of *P. stipitis* to corn cob acid-hydrolyzed hemicelluloses resulted in a significantly higher fermentation rate. Martin et al. [69] showed that a xylose-utilizing genetically engineered strain of *S. cerevisiae* adapted to sugar cane bagasse hydrolysates had increased tolerance to phenolic compounds, furaldehydes, and aliphatic acids. As an alternative to adaptation, genetic engineering can improve microorganisms to better withstand specific inhibitory compounds. However, this can only be attempted if the inhibiting mechanism is known [87].

Carbon catabolite repression can limit the industrial application of co-cultures with xylose-fermenting yeasts, because ethanol produced from glucose may decrease the yield due to the inhibition of the xylose fermentation. As discussed in the previous section, the continuous condition can help to provide an environment with less glucose (or even no glucose) and more xylose at an optimal dilution rate. To overcome the problem related to carbon catabolite repression, Kordowska-Wiater and Targoński [52] applied restricted catabolite repressed mutant *P. stipitis* in their co-culture system.

Developing industrial-level co-culture fermentation processes is crucial for successful application [6]. The simultaneous saccharification and fermentation (SSF) process has been proposed as one of the promising designs for ethanol production, because it can overcome enzyme inhibition and eliminate the need for separate reactors for saccharification and fermentation [67]. A problem arises due to the different temperature and pH optima for hydrolysis and fermentation when balancing co-culture in a SSF process. Utilization of thermophilic anaerobic bacteria combinations (such as the combination of *C. thermocellum* and *C. thermosaccharolyticum*) could be a possible solution, since many thermophilic bacteria produce cellulose and hemicellulase enzymes and have the ability to ferment biomass to ethanol without addition of external hydrolytic enzymes. The strategy of using thermophilic anaerobic bacteria combinations not only can solve the problem of conflict between temperature and pH optima for hydrolysis and fermentation in a SSF process, but also can significantly reduce the enzyme cost for hydrolysis, since hydrolysis and fermentation are completed by the same strain combinations.

Although ethanol production by co-culture fermentation at industrial scale remains problematic, considerable progress has been made by using co-cultivation of different microorganisms for ethanol production at laboratory scale. Harish et al. [38] recently showed that co-culture fermentation of *C. thermocellum* with *C. thermosaccharolyticum* on banana argowaste hydrolysate with maximum ethanol of 0.41 g/g was more efficient in terms of ethanol production, cellulose degradation, and reducing sugars utilization. Co-culture of *Z. mobilis* and *P. stipitis* on a mixture of 30 g/l glucose and 20 g/l xylose and sugarcane bagasse hydrolysate resulted in complete sugar conversion within 19 and 26 h, respectively, with ethanol yield of 0.49–0.50 g/g [26]. This result is one of the best reported so far. Okuda et al. [75] reported co-culture of *E. coli* KO11 with *S. cerevisiae* as one of the methods to increase overall ethanol yield. Patle and Lal [77] described that enzymatic hydrolysis of agricultural waste yielded 97.7% of the theoretical yield of ethanol by co-culture of *Z. mobilis* and *C. tropicalis*. Qian et al. [74] reported a conversion rate of 96.1% within 48 h when using softwood hydrolysate as substrate in co-culture fermentations of *S. cerevisiae* with *P. tannophilus*, and *S. cerevisiae* with a recombinant *E. coli* strain. In these cited examples, the co-culture fermentation process offers the possibility to increase the ethanol yield and production rate, shorten fermentation time, and reduce process costs due to simultaneous fermentation of glucose and xylose. Therefore, co-culture fermentation may have a great impact on the development of a low-cost commercial process for ethanol production.

Future directions

Different strain combinations for co-culture systems and screening methods

The most commonly used combination in current co-culture systems is the pair *P. stipitis* and *S. cerevisiae* or its respiratory-deficient mutant; this pair has better compatibility and better fermentation performance. With respect to the combination of yeast and bacteria, *S. cerevisiae* and *E. coli*, *P. tannophilis* and *Z. mobilis*, *P. stipitis* and *Z. mobilis*, and *C. tropicalis* and *Z. mobilis* are rarely tested. With the exception of the pair *C. thermocellum* and *C. thermosaccharolyticum*, no pairs of bacteria have been used so far. With improvements in genetic engineering and in recombinant DNA technology, more robust glucose- and xylose-fermenting microorganisms should become available. Future research into co-culture for ethanol production could expand the selection of different pairs in co-culture systems, such as *P. stipitis* with recombinant *E. coli*, *C.*

shehatae associated with *K. marxianus*, *C. shehatae* with recombinant *E. coli*, *C. shehatae* with *Z. mobilis*, *P. tannophilis* with *K. marxianus*, *P. tannophilis* associated with *S. cerevisiae* (normal or respiratory deficient mutants), and *P. tannophilis* with recombinant *E. coli*.

In addition, criteria for defining stable co-culture systems have not been established and screening technologies for finding more capable strains for use in co-culture systems are still under development. All of these areas could be future research topics.

Kinetic model for co-culture systems

Diverse kinetic models have been proposed for pure cultures on co-fermentation of glucose and xylose in the literature [8, 40, 64–66, 76, 92]. However, very little investigation has been done on modeling of such a co-culture system to describe the dynamics of the system quantitatively. This is due to the complex nature of the dynamics, the difficulty in analyzing the dynamics, and control of systems containing two microorganisms.

Another problem associated with developing kinetic models for co-culture systems is the lack of kinetic parameters for each strain in a co-culture system. Due to the interactions between the two strains in co-culture systems, the behavior of each strain is different from that seen in pure culture, and therefore the kinetic parameters of each strain in co-culture and pure culture will be different. In theory, by capturing the dynamic properties of systems, a kinetic model could be used as a powerful tool to help obtain optimum operating conditions, achieve sufficient profitability, and reduce tests by eliminating extreme possibilities. For these reasons, it would be worthwhile to develop a kinetic model for ethanol-production co-culture systems.

Metabolic network model for co-culture systems

Metabolic engineering including blocking of undesirable pathways and induction of gene expression associated with desirable pathways to enhance production of ethanol using lignocellulosic biomass is underway. A metabolic network model can help to (1) predict or explain phenotypic behavior *in silico*, (2) identify novel metabolic functions, and (3) design microbial strains for industrial production [13].

A genome-scale metabolic network model can be built for any organism whose genome sequence has been well studied and for which biochemical and physiological information are available. With the completion of the *E. coli* and *S. cerevisiae* genome sequences, construction of genome-scale metabolic network models for *E. coli* and *S. cerevisiae* have been well studied [25, 48, 79, 80, 93–95]. The complete genome of *P. stipitis*, one of the commonly used xylose-fermenting yeasts, has been sequenced and assembled [45]. However, there is no research work about details of the metabolic network model for this microorganism. The knowledge that has been gained from modeling *E. coli* and *S. cerevisiae* could be directly applied to modeling *P. stipitis*, and other microorganisms, such as *Z. mobilis*, *C. shehatae*, and *P. tannophilis*, only if their genome sequences are available. Based on a genome-scale reconstructed metabolic network model for each strain in a co-culture system, a combined metabolic network model could be further developed, which may illustrate the potential metabolic interactions between the two strains. Using the elementary mode and extreme pathway analyses for the combined metabolic network model, one could gain valuable information, such as which genes are essential for producing ethanol, which enzymes would most likely be regulated by changing growth conditions, and what kind of metabolic interactions would appear between the two strains in the co-culture system. Work in this area also is suggested as one of the future directions for co-culture research.

Conclusions

Despite the limited information available for co-culture research, the available examples show that co-culture fermentation could be utilized for ethanol production from lignocellulosic biomass. Co-culture fermentation provides the opportunity to achieve simultaneous conversion of glucose and xylose, maximize substrate utilization rate, increase ethanol yield and production rate, and reduce process costs. However, as a immature but promising technology, application of co-culture fermentation for ethanol production at industrial scale has some challenges. More research efforts need to be applied in this area to develop robust and well-studied co-culture systems that could be used for cost-competitive conversion of biomass to ethanol.

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