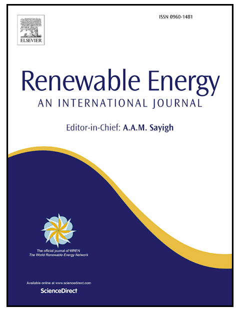

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Review

Lignocellulosic ethanol production: Evaluation of new approaches, cell immobilization and reactor configurations

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Abstract

The environmentally-friendly, economically-viable production of ethanol from cellulosic biomass remains a major contemporary challenge. Much work has been done on the disruption of cellulosic biomass structure, the production of enzymes for the conversion of cellulose and hemicellulose into simple sugars that can be fermented by bacteria or yeast, and the metabolic engineering of ethanol-producing microbes. The results of these studies have enabled the transition from laboratory to industrial scale of cellulosic ethanol production. Notably, however, current processes use free microbial cells in batch reactors. This review highlights the advantages of using immobilized and co-immobilized cells together with continuous bioreactor configurations. These developments have the potential to improve both the yield and the green credentials of cellulosic ethanol production in modern industrial settings.

Keywords:

Cellulosic ethanol, fermentation, co-fermentation, immobilization, immobilized cell reactors

1. Bioethanol production: the search for an economically-viable process

Bioethanol is produced on a global scale to meet the energy requirements of the modern transportation sector; by using renewable resources for ethanol production, the ecological and environmental impact of drilling, transporting and processing fossil fuels could, in principle, be reduced (Nagajaran, et al., 2017) (Aditiya, et al., 2016) (de Azevedo, et al., 2017). Sugar- and starch-based materials such as sugarcane (de Souza Dias, et al., 2015; Duarte, et al., 2013; Rolz & de Leon, 2011), sugar beet (Alexiades, et al., 2016) (Icoz , et al., 2009), corn starch, wheat, rye, barley, cassava (Tran, et al., 2010; Apiwatanapiwat, et al., 2011; Papong & Malakul, 2010) and potato starch (Bo Young, et al., 2008) are the main feedstock for so-called 'first-generation' bioethanol production. The high sugar content of these crops can be converted to bioethanol by microbial fermentation. Since small changes in bioethanol yield have a substantial impact on the economic viability of its production (Gombert & van Maris, 2015), many researchers have also developed microbial strains capable of producing higher ethanol yields than wild-type cultures (Thapa, et al., 2015) (Khramtsov, et al., 2011). Despite these advances, the fact that first-generation bioethanol production uses crops that have been diverted from the food chain has led researchers to seek non-food-based alternatives.

Forest biomass (hard- and softwood and wood chips), the organic fraction of municipal solid waste (MSW), agricultural residues and non-food crops such as switchgrass and alfalfa are all classified as 'cellulosic biomass'. Second-generation bioethanol production from non-food-based, cellulosic biomass comprises four main steps (Naik, et al., 2010): i) biomass pre-treatment to render the cellulose susceptible to hydrolysis; ii) hydrolysis to release simple sugars that can be fermented by bacteria or yeast; iii) microbial fermentation and iv) distillation (Figure 1). Although the composition and the carbohydrate content of cellulosic biomass can differ depending on the biomass sub-type (Table 1), a typical composition is 30-

50% cellulose, 20-40% hemicellulose, and 10-20 % lignin. Xylans are the most abundant hemicellulose component of agricultural lignocellulosic materials. To produce ethanol from such lignocellulosic biomass, the cellulose and hemicellulose must be converted to hexoses and pentoses such as glucose, mannose, arabinose and xylose. Pre-treatment disrupts the biomass structure by removing the lignin that prevents enzymatic or chemical access to cellulose. Efficient and cost-effective methods for the pre-treatment and hydrolyzation of lignocellulosic biomass are needed (Kawaguchi, et al., 2016). Various physical, chemical and biological pre-treatment processes have been developed for this purpose in the last few decades (Aita, et al., 2011) (Alvira, et al., 2010) (Carrasco, et al., 2011) (Chen, et al., 2008). In addition to these processes, new technologies such as thermomechanical instantaneous controlled pressure drop (DIC) pre-treatment has been developed to improve enzymatic saccharification and shorten the pre-treatment duration (Messaoudi, et al., 2015) (Smichi, et al., 2018). The separated lignin can be used as a fuel to run an ethanol plant, but to improve economic feasibility, a portion of the lignin needs to be converted to higher-values chemicals (Wertz, et al., 2018). In order to reduce the cost of production, various strategies such as finding the cheapest renewable source and optimizing process conditions have been assessed (Stephen, et al., 2012) (Wen, et al., 2015) (de Jong, et al., 2017); in these studies, the main economic obstacle to cost-competitive cellulosic biofuel production appeared to be the cost of conversion rather than the cost of the feedstock (Lynd, et al., 2017). Li and Gi (Li & Ge, 2017) developed a system-level cost model for cellulosic biofuel production and investigated the relationships between process characteristics and system performance; they reported that by changing the feedstock particle size, acid concentration, pre-treatment temperature and the duration of the enzymatic hydrolysis and fermentation processes, the total cost could be reduced by 12.8% without any loss in ethanol yield. Production of cellulosic ethanol also generated less CO_2 than fossil fuel sources (Christian, 2015). Even though these studies demonstrate that there is a higher production cost for second- than first-generation bioethanol, this may change as the cost of biomass reduces (Gyekye, 2017).

Wheat and rice are two agricultural crops that are produced world-wide for food and are responsible for generating the majority of lignocellulosic waste biomass. The abundance of these waste materials and their high cellulose and hemicellulose content makes them suitable for ethanol production. Wheat straw, which can produce 104 Gl of bioethanol, is very favourable in Europe (Kim & Dale, 2004). The annual global production of rice straw is 731 million tons and its estimated bioethanol production is 205 Gl. In Asia, 667.6 million tonnes of rice straw are produced annually (Saini, et al., 2015).

Algae are able to metabolize various waste streams (e.g. waste water and carbon dioxide generated by industrial applications) and produce valuable products such as lipids (which can be used for biodiesel production) and carbohydrates (which can be processed to ethanol) (Menetrez, 2012). Furthermore, due to the absence of lignin, algal carbohydrates can be used for bioethanol production after a relatively easy saccharification process (Lee & Lee, 2016). Hence, microalgae have received considerable interest as a potential feedstock for bioethanol production.

Seaweed (macroalgae) have a lower lipid and higher carbohydrate content than microalgae (Nhat, et al., 2018). Similar to microalgae, seaweed do not need land and freshwater for cultivation (Xu, et al., 2014). Besides their usage as a food, different species of seaweed have been used to produce some industrial products, such as alginate, agar, carrageenan and liquid fertilizers. The total industrial consumption of seaweed is greater than 1,500,000 tonnes/year (Jensen, 1993). In 2009, 30,500 tonnes of dry *Laminaria* spp. was harvested only for alginate production (Bixler & Porse, 2011). Ge at. al., (Ge, et al., 2011) reported that, after alginate extraction, the remaining floating residue of *Laminaria japonica* can be used for ethanol production. They reported that, under optimal conditions of dilute sulfuric acid pre-treatment (0.1%, w/w at 21 °C, for 1h) followed by enzymatic hydrolysis (with cellobiase and cellulase at 50 °C, pH 4.8, for 48h), 277.5 mg of glucose (which could be used for ethanol production) was obtained from 1g of floating residue.

The USA and Brazil are the primary producers of bioethanol. In 2009, USA produced 39.5×10^9 l of ethanol using corn while Brazil produced 30×10^9 l of ethanol using sugarcane as a feedstock (Saini, et al., 2015). Since these feedstocks compete with food, they are unsuitable to meet the increasing demand for fuels because of the negative impact on biodiversity (Hahn-Hagerdal, et al., 2006). To produce more sustainable and economical bioethanol, large scale bioethanol production from cellulosic biomass is needed. Biofuel policies in the USA and EU are promoting developments for the generation of cellulosic biofuels worldwide (Gnansounou, 2010). GranBio, a Brazilian biotechnology company constructed the first commercial-scale cellulosic ethanol factory that has a capacity to produce 82 million litres of ethanol per annum from cellulosic feedstock; it started production in September 2014 (GranBio, 2017). The majority of cellulosic ethanol plants in Europe are still at pilot or demonstration stages. Table 2 shows the operational high-capacity of cellulosic ethanol plants in Europe.

During the last two decades, many organisms have been engineered to increase the performance of cellulolytic enzymes required for the hydrolysis step of a second-generation process (Elkins, et al., 2010) (Wu & Arnold, 2013) (Trudeau, et al., 2014). However, a significant effort is still required to lower the cost contribution of cellulolytic enzyme production to the total production cost of bioethanol (Klein-Marcuschamer, et al., 2011). The National Renewable Energy Laboratory (NREL) lowered the cost of cellulosic ethanol from about \$10/gallon to \$2.15/gallon in ten years by enzyme engineering (Christian, 2015). Low enzyme costs can also be attributed to the reasonably-high grants given to the enzyme producers Novozymes and Genencor (now a subsidiary of DuPont) by the US DOE in 2001 (Niiler, 2001). Recently, Lux Research, a US-based technology consultancy firm, investigated the cost of lignocellulosic ethanol production from six different cellulosic feedstocks (corn stover, empty fruit bunches, sugarcane bagasse, sugarcane straw, wheat straw and wood) and three pre-treatment processes (dilute acid, steam explosion and alkali). They concluded that lowering feedstock cost is the most important step in cellulosic ethanol achieving cost parity with first-generation ethanol (Yu, 2016).

Recently, new technologies to fractionate MSW and convert the cheap organic fraction to ethanol have been investigated: following enzymatic saccharification of dilute-acid- and steam-pre-treated biodegradable MSW fractions, Li et al. (Li, et al., 2007) produced glucose from MSW with a yield of 72.80%. Kalogo et al. (Kalogo, et al., 2007) developed a model to estimate the life-cycle energy use of a MSW-to-ethanol facility and reported net fossil fuel energy savings of 397-1830 MJ/MT (Mega Joules per Million Tonnes) MSW compared to net fossil fuel energy consumption of 177-577 MJ/MT MSW for landfilling the waste. Recently, Fiberight LLC, started to produce second generation bioethanol by converting the organic fraction of MSW at industrial scale (Schwab, et al., 2016).

Third-generation bioethanol production uses photosynthetic algae as a feedstock. Unlike lignocellulosic biomass, algal cells contain no or little lignin. However, algal feedstock does require pre-treatment, saccharification and fermentation (Fathima, et al., 2016). Microalgal biomass treated with 0.5 g O_3/per gram dry biomass was used to improve enzymatic saccharification yields; it was reported that 80% of total algal carbohydrate could be converted to glucose using ozone pre-treatment (Keris-Sen & Gurol, 2017). Currently, the conversion of algae to ethanol is still at the development stage (El-Mashad, 2015) (Bin Hossain, et al., 2015).

2. Microorganisms used for cellulosic ethanol production

Microbial fermentation, the main step of bioethanol production, is conversion of sugars into ethanol and carbon dioxide with the help of fermenting microorganisms. The microorganisms used in a fermentation process are selected depending upon the specific carbohydrate content of the biomass. *Saccharomyces cerevisiae*, which is capable of converting glucose to ethanol and is the most commonly-employed yeast in cellulosic ethanol production (Azhar, et al., 2017), cannot convert pentoses to ethanol. Consequently, some other natural yeasts and bacteria capable of fermenting pentoses to ethanol have been used on pentose-rich feedstocks to increase the ethanol yield (Table 3). Pentose-fermenting microorganisms can be used as a pure culture or as a co-culture with hexose-fermenting microorganisms (Karagoz & Ozkan, 2014). Pure cultures and co-cultures can be employed in batch, fed-batch or continuous fermentation processes. Continuous processes are of great importance in the biofuel industry (Skupin & Metzger, 2017) because they can have positive outcomes compared with batch or fed-batch processes (Thani, et al., 2016): ethanol and other by-products are continuously removed meaning that high bioethanol yields can be reached at high concentrations of both cells and carbon source (Santos, et al., 2015).

S. cerevisiae, the yeast most commonly used for fermentation, has been used in bread and beer production since ancient times (Gallone, et al., 2016). *S. cerevisiae* utilizes the fructose diphosphate pathway in order to breakdown glucose, thereby producing two molecules of pyruvate from one molecule of glucose. The net reaction is as follows:

$$Glucose + 2Pi + 2ADP + 2NAD^{+} \rightarrow 2Pyruvate + 2ATP + 2NADH + 2H^{+} + H_2O$$

Lignocellulosic biomass, upon pretreatment and enzymatic hydrolysis, generates a mixture of hexose and pentose sugars such as glucose, xylose, arabinose and galactose (Cotta, 2012). Although *S. cerevisiae* cannot transform xylose to ethanol, in the presence of xylose isomerase, xylose is converted to xylulose, which can be fermented by *S. cerevisiae*. In addition, *Candida shehatea*, *Scheffersomyces stipitis* and *Pachysolen tannophilus* can ferment xylose as part of their natural metabolism (Abbi, et al., 1996). In all cases, these yeasts transform xylose to xylulose, allowing its utilization in ethanol production via the pentose phosphate pathway.

S. stipitis can produce ethanol by fermenting glucose, xylose or cellobiose (a disaccharide consisting of two glucose units in a β1-4 glycosidic linkage obtained from the partial hydrolysis of cellulose), forming few by-products (Hahn-Hagerdal, et al., 1994) (Grio, et al., 2010). Moreover, this yeast species does not require vitamin supplementation (Agbogbo, et al., 2006). Slinger et al. (Slinger, et al., 1990) reported that xylose concentrations above 40 g/L and ethanol concentrations above 64 g/L inhibited the growth of *S. stipitis* cells. *S. stipitis* exhibits a higher affinity for glucose than for xylose (Weierstall, et al., 1999); cells preferentially convert glucose to ethanol (Agbogbo, et al., 2006). Increasing ethanol concentrations in the medium inhibits xylose fermentation (Karagoz & Ozkan, 2014). The oxygen concentration in the medium also influences xylitol production and thus ethanol production (du Preez, 1994); the efficiency of ethanol production by *S. stipitis* cells is enhanced with decreasing oxygen concentration, whereas ethanol production halts in anaerobic conditions because of poor xylose transport (Bruinenberg, et al., 1984) (Ligthelm, et al., 1988). Studies performed under anaerobic conditions did not report the presence of xylitol or ethanol production, but demonstrated that cells could reproduce. In limited oxygen concentrations (microaerobic conditions), cell reproduction was found to be low, but xylitol and ethanol production was observed to increase (Rizzi, et al., 1989) (Laplace, et al., 1991). For yeast species that ferment xylose such as *S. stipitis* and *C. shehatea*, the glucose uptake rate is far greater than the rate of xylose uptake. Therefore, the presence of high glucose concentrations in the medium will inhibit the utilization of xylose until the glucose concentration declines.

Processes that simultaneously use more than one microorganism are often more challenging than ones using single species; this is because of competition between microorganisms that typically have different metabolic requirements. Synchronous fermentation processes using *Zymomonas mobilis* and *S. stipitis* (Fu, et al., 2009) or *S. stipitis* and *S. cerevisiae* (Grootjen, et al., 1990) (Taniguchi, et al., 1997) have been used to produce ethanol from xylose and glucose. *S. stipitis* can efficiently transform xylose to ethanol, while *S. cerevisiae* is pre-eminent in producing ethanol from glucose. For this reason, studies related to the concurrent use of *S. stipitis* and *S. cerevisiae* cells have recently gained popularity (Yadav, et al., 2011) (Wan, et al., 2011) (De Bari, et al., 2013) (Hanly, et al., 2013) (Santosh, et al., 2017) (Ntaikou, et al., 2018).

It is clear that a major technical hurdle to converting lignocellulose to ethanol is finding appropriate microorganisms for fermentation of both hexose and pentose sugars. A number of recombinant microorganisms including *Escherichia coli*, *Klebsiella oxytoca*, *Z. mobilis* and *S. cerevisiae* have been developed over last decades with the goal of fermenting both hexose and pentose sugars to ethanol simultaneously (Cotta, 2012). Cellulolytic, ethanol-producing microorganisms have been also engineered for increasing their ethanol tolerance and yield of ethanol production. *C. cellulolyticum* and *C. thermocellum* strains able to ferment crystalline cellulose to ethanol with yields close to 60% of the theoretical maximum were obtained with genetic modifications. Yeast cells engineered for secretion of free cellulases or the display of a minicellulosome were able to convert crystalline cellulose to ethanol (Argyros, et al., 2011) (Li, et al., 2012) (Fan, et al., 2012). However, for economically sustainable cellulosic bioethanol production with recombinant strains, further progress in metabolic engineering of these microorganisms is needed (Mazzoli, 2012).

3. Can microbial immobilization improve fermentation yields in continuous processes?

Many microorganisms are able to adhere to different surfaces in nature; immobilization is a technique that mimics this phenomenon (Kourkoutas, et al., 2004). In principle, a continuous process that uses immobilized cells will require a lower reaction volume than a batch process, thereby reducing costs (Tran, et al., 2015). Immobilization has been demonstrated to enhance reactor productivity, ease the separation of cells from the bulk liquid and facilitate continuous operation over a prolonged period (Behera & Ray, 2015). Most ethanol production processes are limited by a low ethanol production rate together with recyclability and separation problems with respect to the microorganism being used. In continuous systems, utilization of immobilized cells enables higher cell densities within the bioreactor. Continuous fermentation processes with immobilized cells have the potential to increase ethanol production and reduce production costs (Ivannova, et al., 2011). Several research groups have focused on whole-cell immobilization as an alternative to existing microbial fermentation processes (Karagoz & Ozkan, 2014) (Karagoz, et al., 2009) (Amutha & Gunasekaran, 2001) (Baptista, et al., 2006) (Behera, et al., 2010) (El-Dalatony, et al., 2016).

Support materials such as gels (Ramakrishna & Prakasham, 1999), porous cellulose (Sakurai, et al., 2000), natural sponge (Ogbonna, et al., 2001), agarose (Nigam, et al., 1998), alginate (Grootjen, et al., 1990) and carrageenan (Norton, et al., 1995) have all been investigated for cell immobilization. Table 4 shows examples of immobilization materials used for ethanol production.

Immobilization techniques can be divided into four categories: (i) immobilization on solid carrier surfaces; (ii) entrapment within a porous matrix; (iii) mechanical containment behind barriers; and (4) cell flocculation (aggregation) (Figure 2). Porous gel matrices, such as calcium alginate ($C_{18}H_{24}CaO_{19}$), have been widely used to entrap cells and obtain high biomass loadings for fermentation. Even though the structure of calcium alginate beads can be destabilized in the presence of acid or during the diffusion of gases, such as CO_2, immobilization with calcium alginate beads is one of the most widely-used immobilization techniques for bioethanol production (Duarte, et al., 2013). The immobilization of *S. cerevisiae* has been performed by entrapment in calcium alginate for optimization of ethanol production by varying alginic acid concentration, bead size, glucose concentration, temperature and hardening time (Mishra, et al., 2016). Non-toxic synthetic polymers such as polyvinylalcohol (Nurhayati, et al., 2014) and polyHIPE polymer (synthesized using high internal phase emulsions) (Karagoz, et al., 2009) are alternative candidates for industrial applications. The structure of the support material and the immobilization method influence cell physiology and reproduction, mass transport, product quality, bioreactor design and therefore the process economy (Rychtera, et al., 1987) (Kourkoutas, et al., 2004) (Brányik, et al., 2001) (Brányik, et al., 2005) (Verbelen, et al., 2006). Due to the high cell densities that can be achieved, processes using immobilized cells can be more productive than those using suspension-state cultures. Furthermore, due to diffusion and concentration gradients inside support materials, immobilized yeast cells are more tolerant to ethanol and exhibit a lower degree of substrate inhibition compared with free cells (Qun, et al., 2002). Nicolic et al. (Nikolic, et al., 2010) studied the effect of immobilization on the production of bioethanol from corn meal hydrolyzates. They reported that immobilization of *S. cerevisiae* var. *ellipsoideus* using calcium alginate beads resulted in cells with an elevated tolerance to higher substrate and product concentrations compared with free cells due to diffusion and lower concentrations in the core of the beads. Substrate inhibition was detected at an initial glucose concentration of 200 g/L for immobilized cells, whereas free cells were inhibited at 176 g/L. De Bari et al. (De Bari, et al., 2013) demonstrated that immobilization of *S. stipitis* in a silica-hydrogel increased the relative consumption rate of xylose to glucose 2–6-fold depending on the composition of the fermentation medium. However, the final yields obtained with the immobilized cells were not significantly different from those using free cells. On the contrary, Amutha and Gunasekaran (Amutha & Gunasekaran, 2001) reported that when they used co-immobilized *Saccharomyces diastatitus* and *Zymomonas mobilis* cultures to produce ethanol from liquefied cassava starch, a higher ethanol yield (0.38 g/g) was obtained than with free-state cells (0.33 g/g). Notably, due to the high cellular biomass inside the support material, fermentation processes can be terminated earlier with immobilized cells, meaning that the process duration is shorter. It has also been observed that cells retain their activity during multiple consecutive batches or continuous processes. High functional stability, high cell density, easy separation, and resistance to contamination are the most important advantages of using immobilized cells in a bioreactor (Asenjo & Merchuk, 1995).

4. Immobilized cells in continuous culture

In batch systems, microorganisms are inoculated into a closed vessel containing a defined volume of growth medium. No nutritional support is added and no product is removed until the planned fermentation is complete. After inoculation, the cells replicate at a rate specific to their species. The concentrations of substrates in the growth medium decline, toxic metabolites accumulate and environmental conditions (e.g. pH, oxygen concentration) change over time, which can result in the suppression of microbial growth and fermentation. Classical batch fermentations often suffer nutritional restrictions and therefore low cell densities; optimal cell density is a primary factor in achieving high volume productivity (Ramakrishna & Prakasham, 1999).

In continuous systems, regular input of nutrients and harvesting of cells and products occurs. Substrates are fed into the reactor at a defined concentration and flow rate. The number of cells in the reactor is balanced by their removal from the bioreactor; some may be returned to the vessel if required. Most ethanol production processes are limited by a low ethanol production rate together with recyclability and separation problems with respect to the microorganism being used. In continuous systems, utilization of immobilized cells enables higher cell densities within the bioreactor.

Immobilized cells have been used for ethanol production in different reactor configurations. Figure 3 shows classical reactor configurations for using immobilized cells. A continuous stirred tank bioreactor is a cylindrical vessel with a motor driven central shaft supporting the agitator. Through the sparger, air or other gasses are transferred to the medium. The DO concentration can be adjusted by controlling the stirrer speed. Due to their commercial availability, continuous-stirred tank reactors have been widely used on a laboratory scale. Yatmaz et al. (Yatmaz, et al., 2013) produced ethanol from carob pod extract using immobilized *S. cerevisiae* cells in a stirred tank bioreactor. When they used 2% calcium alginate to immobilize cells, they achieved 46% ethanol production yields in fewer than 24 h and were able to reuse the immobilized cells up to five times. In another study, the self-flocculating yeast strain KF-7 was used for continuous ethanol fermentation of molasses-derived sugars in a stirred tank reactor. The authors operated the bioprocess for more than one month and achieved up to 87% of theoretical ethanol yield and 6.6 g/L/h productivity (Tang, et al., 2010). However, at high agitation rates immobilization materials can be disrupted or destroyed by the physical forces of stirred tank bioreactors.

In a flow-through column reactor, agitation can be ensured by the liquid and gas transfer through a column. A packed-bed reactor consists of a column packed with immobilized materials through which medium flows continuously over these matrices. Compared to stirred tank bioreactors, flow-through column and packed-bed reactors have poor mixing conditions. It is rather difficult to control the pH of packed bed bioreactors by the addition of acid or alkali. However, these configurations are preferred for bioprocessing technology involving product-inhibited reactions (Jha, 2017) such as ethanol production; they are the most studied processes employing immobilized cells in the literature (Table 4). In packed-bed and fluidized-bed reactors, substrate passes through the immobilized cells at a constant rate. Such reactors have advantages including ease of running and high reaction rates. Particle catalysts that are placed in the reactor have a highly-specific surface area for solid-liquid interaction

(Asenjo & Merchuk, 1995). With such reactors, it is possible to achieve good interactions between the solid and liquid phases and a reversible system when heat and mass transfer are required. Unlike suspended systems, highly-dense cell concentrations can be achieved. Packed-bed reactors have been used to produce ethanol in a continuous system using *S. cerevisiae* immobilized on a calcium alginate bed (Linko & Linko, 1981) or a microporous hydrophobic polymer matrix (Karagoz, et al., 2009). Yatmaz et al. (Yatmaz, et al., 2013) immobilized *S. cerevisiae* cells on calcium alginate beads in a stirred tank bioreactor and produced 40.19 g/L ethanol from carob pod extract at 3.19 g/L/h. In another study, *Kluyveromyces marxians* cells entrapped with calcium alginate were used to produce ethanol from whey permeate in a continuous fluidized-bed reactor at a dilution rate of 0.3 h^{-1}; 6.01 g/L/h ethanol was produced (Sabrina, et al., 2014). Table 5 shows the ethanol productivities and process conditions of previous studies performed with different support materials and organisms. Higher ethanol productivities are observed with the use of novel support materials in immobilized cell reactors.

A rotating bed bioreactor has a similar structure to a stirred-tank bioreactor. A basket that separates the immobilized material from the culture medium spins on a central shaft. Rotating bed bioreactors have good fluid mixing conditions and are associated with lower mechanical and hydrodynamic shear stresses compared to stirred-tank bioreactor (Reichardt, et al., 2013).

Despite their potential to provide high mass transfer efficiencies, rotating-bed bioreactors have not been widely used in bioethanol production. Early studies using this reactor configuration produced ethanol at a dilution rate of 0.3 h^{-1}, giving an ethanol productivity of 7.1 g/L/h (Del Borghi, et al., 1985). However, more recent studies on this reactor configuration have focused on bioprocesses using immobilized enzymes (Sheelu, et al., 2008) (Wang, et al., 2011) (Xu, et al., 2017).

Co-fermentation can be easily performed by the immobilization of two or more different strains capable of fermenting different sugars. Different cultures can be co-immobilized together on the same support material or separately on different materials meaning that the different environmental needs of different strains can be satisfied in the same vessel. Even though mixed cultures are widely used in biofuel production (Antonopoulou, et al., 2008), only a few studies have focused on ethanol production with co-immobilized cultures (Grootjen, et al., 1990) (Pornkamol & Friedrich, 2010). Even fewer studies have investigated co-immobilized cells in continuous bioreactors (Unrean & Srienc, 2010) (de Almeida & de Franceschi de Angelis, 016) (Karagoz & Ozkan, 2014). However, the success of these studies suggests the potential of this approach (Chen, 2011).

Grootjen et al. (Grootjen, et al., 1990) trapped *S. stipitis* cells within alginate beads and evaluated their fermentation capacity in a medium composed of glucose and xylose with free *S. cerevisiae* cells. Due to mass transfer restrictions, *S. stipitis* cells trapped in alginate beads experience reduced local glucose concentrations and therefore consume xylose. This same co-immobilization strategy has been used to produce ethanol from wheat straw hydrolysate in a packed-bed reactor. The ethanol productivity of co-immobilized *S. cerevisiae* and *S. stipitis* was compared with individually immobilized *S. cerevisiae* and *S. stipitis* cells. The study showed that higher ethanol production rates could be achieved by using co-immobilized *S. cerevisiae* and *S. stipitis* and that 73.92% of the xylose in the hydrolysate was consumed to produce 41.68 g/L day ethanol at a hydraulic retention time (HRT) of 6 h (Karagoz & Ozkan, 2014). In another study (Pornkamol & Friedrich, 2010), ethanologenic *E. coli* strains developed to selectively consume pentoses or hexoses were immobilized and co-immobilized in calcium alginate beads. It was reported that 2.2 g/L.h ethanol was produced by co-immobilized cells, which is higher than the ethanol production rate (1.6 g/L.h) obtained from single cultures.

5. Challenges for large scale ethanol production with immobilized cells in continuous processes

A variety of immobilized cell bioreactors has been developed to optimize fermentation processes. Immobilized cells are currently being used industrially for vinegar, organic and amino acid production, as well as in wastewater treatment (Zhu, 2007). There are also successful applications of immobilized systems in the dairy industry (Koutinas, et al., 2009) (Champagne, et al., 1994) (Groboillot, et al., 1994),

Verbelen et.al. (2006) reviewed continuous ethanol production with immobilized yeast cells for beer production. The first continuous fermentation system appeared in the 1960s, but few systems grew up to industrial scale, indicating technical and qualitative pitfalls associated with this technology (Verbelen et al., 2006). Gas lift and packed bed reactors were used for the purpose of beer fermentation in continuous systems. It is reported that continuous ethanol production processes may create some problems for beverage production, since preventing contamination and keeping flavour quality are important issues for this industry. Branyik et.al., (2005) reviewed continuous fermentation systems based on immobilized cell technology for beer production. They noted that immobilized cell systems were condemned to failure for several reasons including engineering problems associated with excess biomass, problems with CO_2 removal, optimization of operating conditions and clogging and channelling of the reactor. However, design of new reactors, understanding the behaviour of immobilized cells and applications of novel carrier materials, provided a new stimulus to improve and apply immobilized cell systems at an industrial scale (Branyik, et al., 2005).

Although production of alcoholic beverages is not a subject of this review, the obstacles and challenges are very similar in the bioethanol and dairy industries in terms of the use of immobilized cells for production. Moreno Garcia et al., (2018) discuss future perspectives for yeast cell immobilization for alcoholic wine fermentations. They reported that there are not many applications for winemaking at an industrial level. Difficulty in upgrading, inefficient adherence of the cells to current immobilization materials, investment problems and a lack of knowledge on the use of immobilized yeasts for alcoholic fermentation are listed as reasons. Novel and cheap immobilization materials are regarded as a main solution for the production of ethanol using immobilized systems. One novel technology is the use of filamentous fungi as an immobilization material (Garcia Martinez et al 2011). Ethanol fermentation for the transportation sector may benefit from continuous ethanol production technologies since some requirements, such as aroma quality, are not a problem for the lignocellulosic bioethanol production sector.

Use of immobilized cells in industrial processes has great potential to eliminate continuous centrifugation for cell recycling, which can bring additional savings in the construction and operation of industrial units. As outlined in this review with examples from laboratory scale studies, the use of continuous systems with immobilized yeasts could achieve more economical bioethanol production in industry. There are few examples of the use of continuous ethanol production in industry (Xie et al., 1999; Carvalho Neto et al., 1990). Vasconcelos et. al. (2004) studied ethanol production with yeast cells immobilized on sugar cane stalks at pilot scale. They reported that continuous immobilized cell reactors allow working with high dilution rates which increases productivity.

Chang et.al. (2014) used sweet sorghum bagasse as an immobilization carrier for acetone-butanol-ethanol fermentation by *Clostridium acetobutycum.* They reported that the fermentation period of the immobilized cell system was almost 28.4% shorter and the productivity was 1.68 times higher than a free cell system (Chang, et al., 2014). Similarly, Diez-Antolinez et.al (2018) screened different yeast and immobilization materials for ethanol production from cheese whey permeate. They reported that Glass Rasching rings and alumina beads showed stable performance over 1,000 hours, yielding ethanol titers of 60 g/L, which substantially reduced yeast cultivation costs (Diez-Antolinez, et al., 2018). The economic benefits associated with cell immobilization and recycling, such as increased yields and productivities and lower capital costs due to shorter residence times should encourage researchers to do further, detailed techno-economic analyses. In the literature, there is a current scarcity of economic analyses comparing free and immobilized cell systems. Mussatto et.al. (2015) used SuperPro Designer v8.5 simulation software to evaluate and compare the economic aspects of free and immobilized cell fermentations for fructooligasaccharide (FOS) production. When they calculated the profit margin for per kg of FOS produced, they found a 25.8% higher profit margin value for immobilized cell systems and lower fermenter, centrifuge and filtration costs. Furthermore, they compared key economic parameters such as the return of investment, payback time and net present value, reporting that immobilized systems are economically more advantageous than free cell systems (Mussatto, et al., 2015). Although there are many reports on the advantages of cell immobilization and few techno-economic analyses supporting their use, it must be noted that the great majority of studies on immobilized cells have been performed at laboratory scale (Ivannova, et al., 2011). Limitations on the application of immobilized cell systems on an industrial scale are mainly attributed to mass transfer limitations within the supports (Zur, et al., 2016). Separation and reuse of immobilized cells is not the only concern for large scale processes; porous structures of some matrices may cause diffusion of the pollutant and various metabolic products into the matrix, which limits continuous reuse of the matrices (Bayat, et al., 2015).

Inadequate immobilization may negatively affect process yields and economics. The type of support material, amount of the cells, concentration and quality of nutrients and temperature and hydraulics of the system are the most important parameters affecting the immobilization of cells (Zacheus, et al., 2000). Desorption of cells reduces product purity, while growth of aerobic cells may be inhibited after immobilization (Wang, et al., 2018). Some immobilization methods, such as entrapment, allow high mechanical strength, but also have disadvantages such as cell leakage and diffusion limitations (Martins, et al., 2013). As an alternative to the entrapment of whole cells into alginate beads, a recently-developed concept of 'teabag catalysis', entrapping cells into containers of polyvinylidene difluoride membrane (cut-off 0.2 µm) inside a spin column reactor has shown high recyclability even under challenging micro-aqueous conditions (Wachtmeister & Rother, 2016).

For bioethanol production, the effect of feedstocks and pre-treatment technologies on techno-economics has been widely studied (Tao, et al., 2011) (Dickson, et al., 2018) (Mupondwa, et al., 2018). However, in the literature, there is lack of detailed cost analysis on immobilized cells and process types for bioethanol production. As outlined above there are many factors to be considered which may prevent investment into immobilized cell systems at an industrial scale. To make a realistic economic comparison of free state versus immobilized cells, each process should be evaluated individually to allow the consideration of all relevant parameters including fermentation type (continuous or batch systems), reactor configuration, type of matrix and the microorganisms used for fermentation.

6. Conclusion

The conversion of cellulosic biomass to ethanol has been studied in depth over the last decades (Aditiya, et al., 2016). Various pre-treatment techniques (Mosier, et al., 2005), different enzyme cocktails (Klyosov, 1990) and genetically engineered cells (Abreu-Cavalheiro & Monteiro, 2013) have been used on a wide range of non-food-based biomass to produce bioethanol. Despite these improvements, cellulosic bioethanol production cannot yet compete economically with fossil fuel production.

Improving fermentation performance by ensuring optimum mass transfer conditions is still a significant challenge (Verbelen, et al., 2006). Immobilization and co-immobilization of cells show great potential for cellulosic ethanol production due to high productivity rates, lower contamination risks and stability of the resultant cultures. Mass transfer limitations and heterogeneous environmental conditions inside a support material generate a new solution to work with mixed cultures with different characteristics. Co-immobilization of mixed cultures converting hexoses and pentoses to ethanol in a matrix may be the key to solve one of the most important issues in cellulosic ethanol production. Literature reports suggest that by using immobilized or co-immobilized cultures in continuous bioreactors, efficient and rapid conversion of mixed sugars to ethanol can be achieved. To sustain optimum conditions for different cultures concurrently, different supports or customized heterogeneous materials can be used. Although there are still some obstacles for large scale bioethanol production by immobilized cells in continuous reactors, efforts should be concentrated on improving this technology, which will contribute to next-generation biorefineries and industrial cellulosic ethanol production plants.

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Figure Captions

Figure 1. Process flow diagram for cellulosic ethanol production, from the beginning (biomass) to the end (fuel)

Figure 2. Whole cell immobilization methods: adsorption, electrostatic binding, covalent binding, entrapment, self-flocculation and mechanical containment (adapted from (Kourkoutas, et al., 2004))

Figure 3. Different types of bioreactors suitable for immobilized cells: *1*- stirred tank reactor, *2*- flow-through column reactor, *3*- fixed-bed column reactor, *4*- rotating-bed reactor

Table Captions

Table 1. Carbohydrate content of typical cellulosic biomasses

Table 2. Operational cellulosic ethanol plants in Europe, adapted from (Bacovsky, et al., 2013)

Table 3. Microorganisms that have high potential for cellulosic ethanol production (adapted from (Zabed, et al., 2016))

Table 4. Immobilization materials used for ethanol production

Table 5. Immobilized cell reactors used for ethanol production

Table 1. Carbohydrate content of typical cellulosic biomasses

Biomass	Cellulose content (%)	Hemicellulose content (%)	Lignin content (%)	Reference
Alfalfa	30.4-31.1	17.6-17.7	13.3-14.5	(Dien, et al., 2011)
Barley straw	36.6-39.1	21.1-25.7	15.2-22.4	(Yang, et al., 2015) (Duque, et al., 2014)
Corn stover	37.0-37.5	18.5-28.9	19.4-22.1	(Saha, Qureshi, Kennedy, & Cotta, 2015) (Yu, et al., 2016)
Grass	31.85-38.51	31.13-42.61	3.10-5.64	(Wongwatanapaiboon, et al., 2012)
Hardwood stems	40.0-55.0	24.0-40.0	18.0-25.0	(Sun & Cheng, 2002)
Microalgae	50-7.3*		n.a.	(Rodjaroen, Juntawong, Mahakhant, & Miyamoto, 2007) (Kim, et al., 2006)
Organic fraction of MSW	57**		n.a.	(Nwobi, et al., 2015)
Rapeseed straw	37.0-44.6	19.6-20.0	18.0-20.0	(Lu, Zhang, & Angelidaki, 2009) (Karagoz, Rocha, & Ozkan, 2012)
Rice straw	38.4-42.54	21.8-24.51	9.16-16.2	(Zhu, et al., 2015) (Akhtar & Goyal, 2017)
Rye straw	33.12-37	22.24-40	19.8-22	(Sun & Cheng, 2002) (Smuga-Kogut, et al., 2017)

Seaweed	30.0***	2.2***	n.a	(Ge, Wang, & Mou, 2011)
Softwood stems	45.0-50.0	25.0-35.0	25.0-35.0	(Sun & Cheng, 2002)
Sugarcane bagasse	43.02-50.43	18.95-25.20	17.02-22.87	(Santosh, Ashtavinayak, Amol, & Sanjay, 2017)
Switchgrass	28.24-35.13	20.25-26.96	15.46-21.15	(Dougherty, et al., 2014) (Keshwani & Cheng, 2009)
Wheat straw	30.2-48.57	22.3-27.70	8.17-17.0	(Saha, Iten, Cotta, & Wu, 2005) (Ballesteros, Negro, Oliva, Cabanas, & Manzanares, 2006)

*Starch content after oil extraction

**Glucan content of total solid

***Composition of floating residue after alginate extraction process

n.a. indicates data are not available.

Table 2. Operational cellulosic ethanol plants in Europe, adapted from (Bacovsky, Ludwiczek, Ognissanto, & Worgetter, 2013)

Company	**Location**	**Plant type**	**Start-up**	**Feedstock**	**Output (t/y)**
Aalborg University	Bornholm (Denmark)	Pilot	2009	Wheat straw	11
Abengoa	Babilafuent (Spain)	Demo	2008	Straw and municipal residues	400
Beta Renewables	Crescentino (Italy)	Commercial	2013	Wheat straw	60,000
BioAgra	Goswinnowice (Polad)	Demo	2014	Wheat straw and corn stover	50,000
ECN	Petten (Netherlands)	Pilot	2008	Clean wood and demolition wood	346
Inbicon	Kalundborg (Denmark)	Demo	2009	Wheat straw	4300
PROCETHOL 2G	Pomacle (France)	Pilot	2011	Woody and agricultural by-products, residues, energy corps	2700
SEKAB/EPAB	Ornskoldsvik (Sweeden)	Pilot	2004	Wood chips and agricultural wastes	160
TNO	Zeist (Netherlands)	Pilot	2002	Wheat straw, grass, corn stover, bagasse, wood chips	100
Weyland AS	Bergen (Norway)	Pilot	2010	Various feedstock, mostly spruce and pine	158

Table 3. Microorganisms that have high potential for cellulosic ethanol production (adapted from (Zabed, Sahu, N, & Faruq, 2016))

Microorganism	**Characteristics**	**Contribution**	**Major feature**
Candida shehatae	Facultative anaerobic yeast	Fermentation	• Able to ferment xylose • Rapid xylose conversion
Clostridium thermocellum	Anaerobic thermophilic bacteria	Fermentation and hydrolysis	• Produces cellulases and hemicellulases and converts cellulosic biomass to sugar • Direct production of ethanol from cellulose
Pachysolen tannophilus	Facultative anaerobic yeast	Fermentation	• Able to ferment xylose
Saccharomyces cerevisiae	Facultative anaerobic yeast	Fermentation	• Robust and well-studied microorganism • Studied to ferment various lignocellulosic hydrolysates • High ethanol yield • Good tolerance to inhibitors and osmotic pressure
Shefferomyces stipitis (*Pichia stipitis*)	Facultative anaerobic yeast	Fermentation	• Efficient conversion of xylose to ethanol • Low by-product formation
Zymomonas mobilis	Gram negative bacterium	Fermentation	• Higher ethanol productivity, compared to *S. cerevisiae* • Low biomass yield and high ethanol yield

Table 4. Immobilization materials used for ethanol production

Immobilization Material	Immobilized culture	Substrate	Yield (g/g)	Reusability	Fermentation type	Fermentation time	Reference
Calcium alginate	*Saccharomyces cerevisiae var ellipsoideus*	Corn meal	0.55	n.a.	Batch fermentation in flasks	38 h	(Nikolic, Mojovic, Rakin, & Pejin, 2009)
	Saccharomyces cerevisiae	Mahula flowers	0.48	Min. 3 cycles	Repeated batch fermentation in flasks	96 h	(Behera, Kar, Mohanty, & Ray, 2010)
	Saccharomyces cerevisiae	Cane molasses	0.46	n.a.	Continuous fermentation in 5x90 cm tubular column reactor	25 days	(Ghorbani, Younesi, Sari, & Najafpour, 2011)
Mesoporous silica	*Zymomonas mobilis*	Glucose	0.47	Min. 10 cycles	Repeated batch fermentation in flasks (500 ml working volume)	24 h*	(Niu, et al., 2013)
Pectin beads	*Zymomonas mobilis*	Glucose	0.45	n.a.	Continuous fermentation in 350 ml expanded bed column reactor	16 h	(Kesava, Panda, & Rakshit, 1995)
Plastic-composite supports	*Saccharomyces cerevisiae*	Glucose	0.5	n.a.	Repeated batch and continuous fermentation in a biofilm reactor with a total external surface	60 days	(Demirci, Pometto, & Ho, 1997)

					area of 60 cm^2		
Poly(vinyl alcohol) cryogel	*Pachysolen tannophilus*	Crude glycerol	0.46	Min.16 cycles	Repeated batch fermentation in flasks (100 ml working volume)	15-24 h*	(Stepanov & Efremenko, 2017)
Wild sugarcane stalks	*Saccharomyces cerevisiae*	Wild sugarcane	0.43	Min. 8 cycles	Repeated batch fermentation in flasks (300 ml working volume)	36 h*	(Chandel, Narasu, Chandrasekhar, Manikyam, & Rao, 2009)

*Fermentation time in each batch

n.a. indicates data are not available.

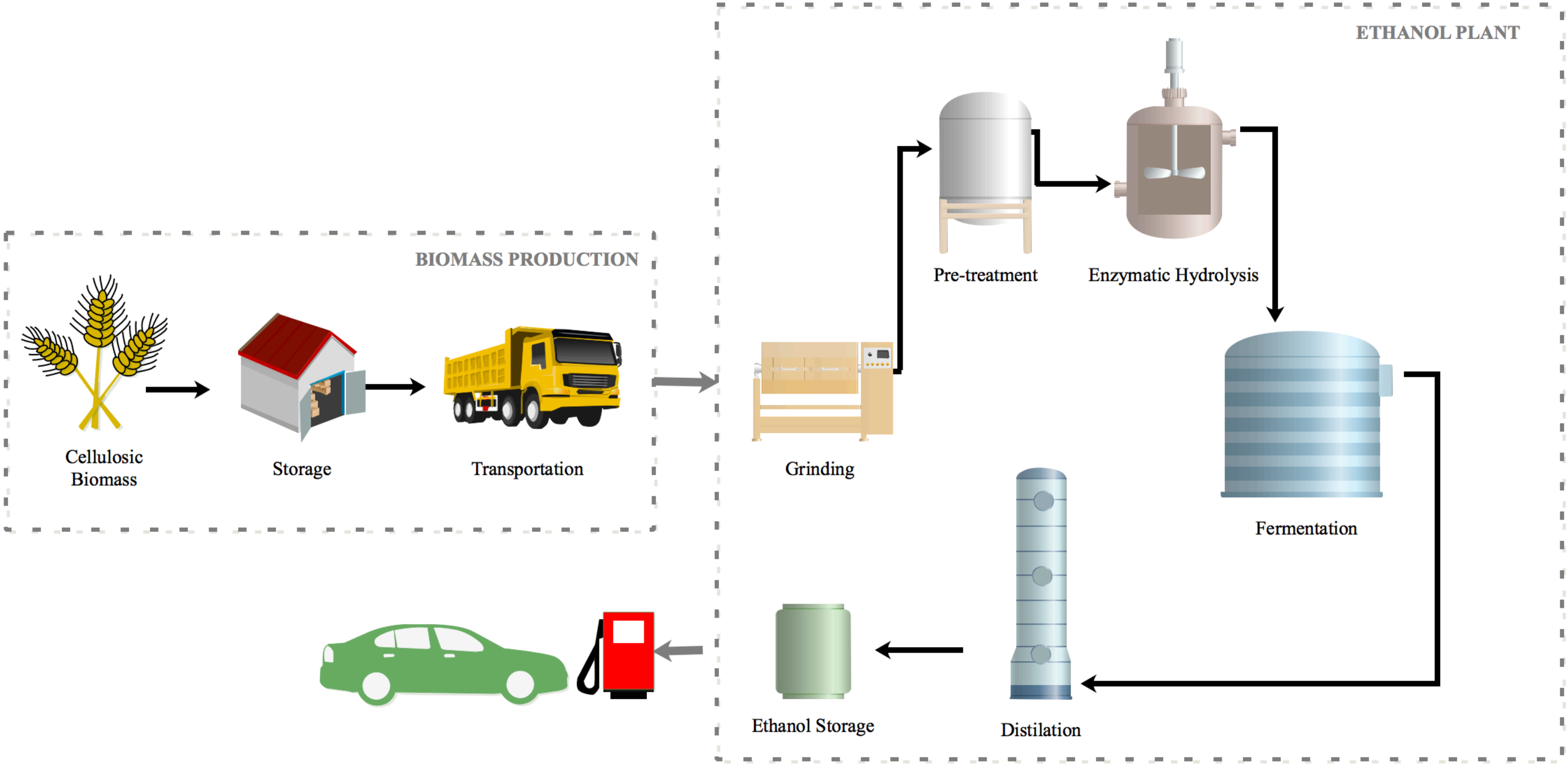
1 Table 5. Immobilized cell reactors used for ethanol production

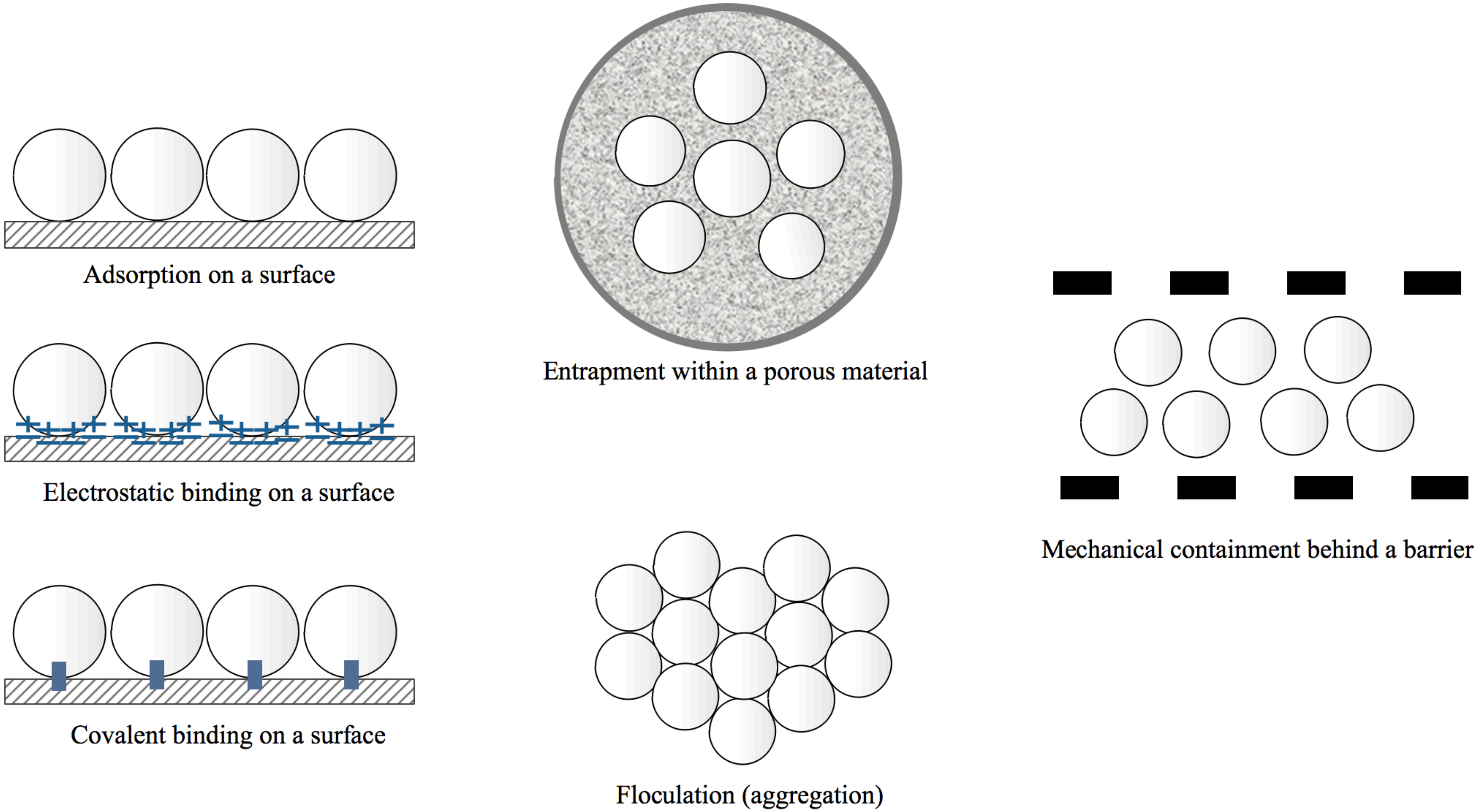
Feedstock	Sugar concentration (g/L)	Immobilization support	Immobilized microorganism	Process/ Reactor type/Working volume	Dilution rate (1/h)	Effluent ethanol concentration (g/L)	Ethanol productivity (g/L/h)	Reference
Acid-pretreated bagasse	20	Polyvinyl alcohol	*Zymomonas mobilis*	Batch/flask/250ml	-	5.53	1.31	(Wirawan, Cheng, Kao, Lee, & Chang, 2012)
		Calcium alginate				5.44	1.27	
Crude glycerol	25	Polyvinyl alcohol cryogel	*Pachysolen tannophilus*	Continuous/flow-through column reactor/850ml	0.062	8.2	0.63	(Stepanov & Efremenko, 2017)
Diluted waste molasses	180	Self-flocculation	*Saccharomyces cerevisae* KF-7	Continuous/stirred tank reactor/2000ml	0.083	80	6.6	(Tang, et al., 2010)
D-xylose	50	Alginate beads treated with $Al(NO_3)_3$	*Clavispora opuntiae*	Continuous/packed-bed reactor/350ml	0.31	9.49*	3.10	(Nigam, Mandal, & Singh, Continuous Ethanol Production from D-xylose II Using Immobilized Cells of Clavispora

								opunitae, 2015)
Glucose	100	Polyurethane foam cubes	*Saccharomyces cerevisiae*	Continuous/fluidised-bed column reactor/1000-5000ml	0.4	40	16	(Baptista, et al., 2006)
Glucose	125	Fe_2O_3-modified polyvinyl alcohol	*Zymomonas mobilis*	Bespoke continuous fermenter/200ml	0.5	62.18	31.09	(Nurhayati, Cheng, Nagarajana, & Chang, 2016)
Glucose and xylose	91	κ-carrageenan	*Zymomonas mobilis*	Continuous/fluidised-bed column reactor/900ml	0.5	30.5	15.3	(Krishnan, Blanco, Shattuck, Nghiem, & Davison, 2000)
Microalgal biomass	22.25	Calcium alginate	*Saccharomyces cerevisiae*	Repeated-batch/ flask/270ml	-	9.7	0.22	(El-Dalatony, et al., 2016)
Oilseed rape straw hydrolysate	60	Lentikat ® discs	*Saccharomyces cerevisiae*	Continuous/ packed-bed column reactor/69ml	0.5	25.8*	12.88	(Mathew, Crook, Chaney, & Humphries, 2014)
Pineapple cannery waste	82.3	κ-carrageenan	*Saccharomyces cerevisiae*	Continuous/ packed-bed reactor/350ml	1.5	28.5	42.8	(Nigam, Continuous ethanol production from pineapple cannery waste using immobilized

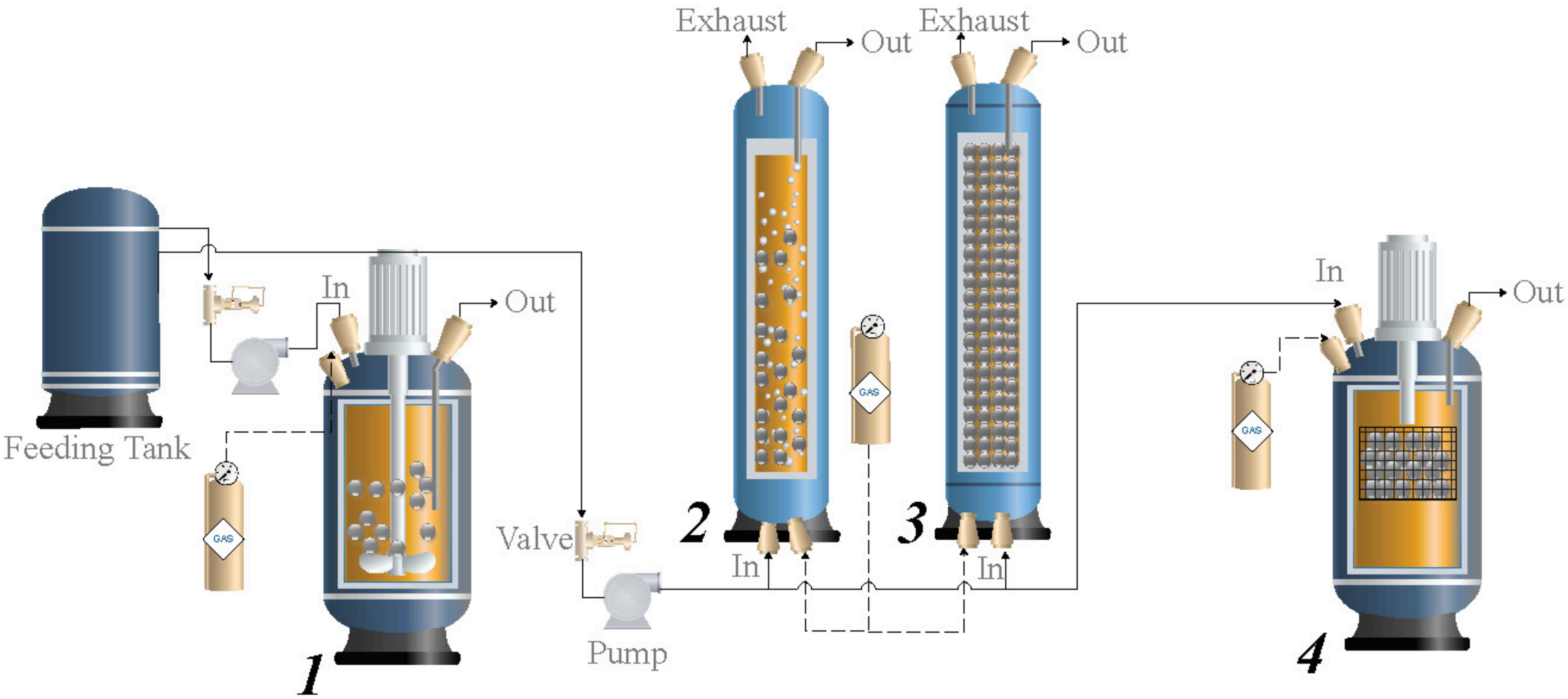
							yeast cells, 2000)
Wheat straw hydrolysate	30	Calcium alginate	*Saccharomyces cerevisiae and Shefferomyces stipitis*	Continuous/packed-bed reactor/180ml	1.333	10.42	9.8	(Karagoz & Ozkan, 2014)

* Data produced from paper


ETHANOL PLANT
BIOMASS PRODUCTION
Cellulosic Biomass
Storage
Transportation
Grinding
Pre-treatment
Enzymatic Hydrolysis
Fermentation
Distilation
Ethanol Storage



Adsorption on a surface
Electrostatic binding on a surface
Covalent binding on a surface
Entrapment within a porous material
Floculation (aggregation)
Mechanical containment behind a barrier



Feeding Tank
In
Out
1
Valve
Pump
Exhaust
Out
Exhaust
Out
2
In
3
In
In
Out
4
GAS
GAS
GAS
GAS

-Cellulosic ethanol production needs application of new technologies for competing with gasoline

- Cell immobilization technologies improve bioethanol productivity

-Ethanol yield in different processes is affected by the reactor configurations