Review

Biofpr Biofuels Bioproducts & Biorefining

Yeast immobilization systems for second-generation ethanol production: actual trends and future perspectives

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Received December 09 2020; Revised April 21 2021; Accepted May 14 2021;
View online May 31, 2021 at Wiley Online Library (wileyonlinelibrary.com);
DOI: 10.1002/bbb.2250; *Biofuels, Bioprod. Bioref*. 15:1549–1565 (2021)

Abstract: Yeast immobilization with low-cost carrier materials is a suitable strategy to optimize the fermentation of lignocellulosic hydrolysates for the production of second-generation (2G) ethanol. It is defined as the physical confinement of intact cells to a certain region of space (the carrier) with the preservation of their biological activity. This technological approach facilitates promising strategies for second-generation bioethanol production due to the enhancement of the fermentation performance that is expected to be achieved. Using immobilized cells, the resistance to inhibitors contained in the hydrolysates and the co-utilization of sugars are improved, along with facilitating separation operations and the reuse of yeast in new production cycles. Until now, the most common immobilization technology used calcium alginate as a yeast carrier but other supports such as biochar or multispecies biofilm membranes have emerged as interesting alternatives. This review compiles updated information about cell carriers and yeast-cell requirements for immobilization, and the benefits and drawbacks of different immobilization systems for second-generation bioethanol production are investigated and compared. © 2021 The Authors. *Biofuels, Bioproducts and Biorefining* published by Society of Industrial Chemistry and John Wiley & Sons Ltd.

Key words: yeast immobilization; yeast; alcoholic fermentation; second-generation ethanol

Introduction

The extensive use of fossil fuels in recent decades has led to their rapid depletion, which has caused concerns about energy security and abnormal increases in greenhouse gases.[1–5] In an increasingly saturated global society, where the transport sector contributes to more than 40% of total fossil fuel consumption, it has been estimated that the reserves of fossil fuels will be consumed in the next 40 to 50 years.[6] With this in mind, the development of alternative renewable fuel sources with a reduced carbon footprint is a priority.

Liquid biofuels, such as bioethanol, biodiesel, or biocrude oil, are produced from renewable materials of plant or animal origin. As liquid biofuels have high calorific value, standard

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Corrections added on 14 June 2021, after first online publication.

transport and storage requirements, and similar properties to gasoline, diesel, or other petroleum-derived energy carriers, they have the potential eventually to replace current transport fossil fuels without major technical modifications to engines and delivery infrastructure.[7–10] Apart from being a technically realistic solution to fossil fuel depletion, the use of liquid biofuels could lead to a substantial reduction of greenhouse gas emissions in transportation.[11,12]

Since the pioneering efforts in Brazil in the early 1970s, bioethanol has been the biofuel that has received most worldwide attention from both academic research and commercial activity.[13] Although today's ethanol production is heavily dependent on first-generation technologies, mostly from corn starch and cane sugar, the second-generation (2G) approach is continuously gaining interest.[14,15] Second-generation ethanol is produced from non-food biomass, such as agricultural and forest residues, non-edible crops, or municipal solid waste. Lignocellulosic ethanol is one of the dominant 2G biofuels, and its combustion generates low greenhouse gas emissions due to its oxygenated nature.[8,16] However, to obtain biofuels from lignocellulosic biomass, a complex four-step process needs to be performed: (i) pretreatment of raw material; (ii) saccharification or hydrolysis of the derived polymers to fermentable monomeric sugars; (iii) fermentation of the sugars to biofuel molecules, and (iv) recovery and purification (Fig. 1). For the rational utilization of lignocellulose, 2G ethanol should be produced following a biorefinery philosophy that includes diversion of all by-products and side streams to other products of high economic and societal value. According to the International Energy Agency (IEA) Bioenergy Task 42, a biorefinery is 'the sustainable processing of biomass into a spectrum of marketable products and energy'.[17]

The complexity of the 2G bioethanol process and difficulties such as the release of inhibitory by-products during pretreatment, or the complex composition of lignocellulosic hydrolysates, obstruct the large-scale implementation of the technology (Fig. 1).[18–20]

Due to degradation reactions occurring during pretreatment, the first step in a sequential biorefinery processing scheme, lignocellulosic hydrolysates contain toxic compounds that inhibit cell growth and ethanol production.[21] Some classic examples of inhibitory compounds are furan aldehydes, aliphatic acids, phenolic compounds,[18] or the more recently discovered quinones, small aliphatic aldehydes, and specific phenols that have greater toxicity than formerly known inhibitors.[22] The concentration of inhibitors in hydrolysates can be decreased by detoxification, but it requires a separate step, which increases the process cost. Increasing the inoculum size can alleviate the inhibition but it also imposes economic restrictions.[23] Other alternatives are a selection of inhibitor-tolerant microbial strains, evolutionary engineering, or metabolic engineering.[18,24] However, even though the engineering of microbial strains enhances their

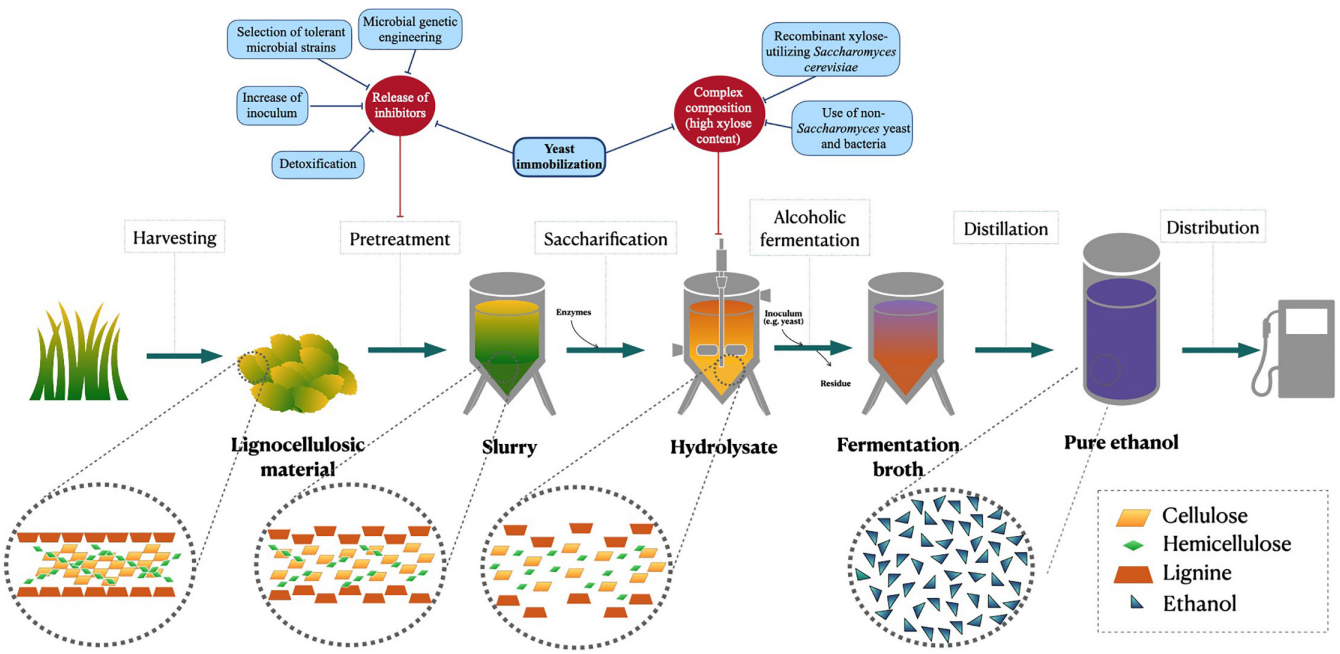

Figure 1. Second-generation bioethanol production, bottlenecks, and potential strategies for minimizing their effects.

resistance to inhibitors, their use implies some drawbacks such as the instability of genetic modifications, regulations for their utilization, or a weaker enhancement than the chemical detoxification methods.[18]

The complex composition of lignocellulosic hydrolysates, which in addition to hexoses also contain pentoses, mainly xylose, is also highly challenging for 2G ethanol-producing microbes. *Saccharomyces cerevisiae*, the most industrially relevant ethanologenic organism, lacks the natural ability to utilize pentoses, and the microorganisms with that ability are not inhibitor tolerant and generally result in a low ethanol yield. Developing recombinant xylose-utilizing strains of *S. cerevisiae* is an issue of major relevance, and several strategies, such as heterologous expression of xylose reductase and xylitol dehydrogenase genes, have been applied in that direction.[25] Despite these efforts, efficient xylose-to-ethanol conversion by *S. cerevisiae* is still challenged by different redox cofactor preferences of the expressed oxidoreductases,[26] xylitol accumulation, and uncertainties about xylose transport system among other limitations.[27] Other strategies considered for further research towards consolidated bioprocessing (CBP) for 2G ethanol production are the use of non-*Saccharomyces* yeasts with industrially relevant properties, such as thermal and ethanol tolerance, as well as targeting thermophilic and cellulolytic bacteria developing efficient ethanol producers.[28–30]

The immobilization of microbial cells can be considered a suitable and viable strategy for dealing with the problems discussed above, and it has been proposed as a viable alternative to optimize the fermentation step to produce lignocellulose-based biofuels (Fig. 1).[31–33] Yeast immobilization is defined as the physical entrapment of active, intact cells into a certain area without affecting their biological activity. The evaluation of the effectiveness of yeast immobilization for enhancing the tolerance to lignocellulose-derived inhibitors,[34] and for improving sugar co-utilization, has been reported.[35] It has recently been shown that immobilization and reutilization of xylose-fermenting *S. cerevisiae* recombinants is promising for achieving cost-effective ethanol production from non-detoxified hydrolysates.[36,37] Besides the previously mentioned, cell immobilization technologies provide benefits such as the increasing cell density, promoting better control of the yeast cells for continuous fermentation, or cell recovery/reutilization;[38,39] which in turn lower the complexity of the 2G ethanol production and improves its economics.[40]

In the context of energy and ecological transition, there is an urgent need to develop new technologies to exploit renewable sources efficiently. With this in mind, the aim of this review is to summarize and compare the latest information related to yeast immobilization technologies investigated for the production of 2G bioethanol, especially regarding (i) the required and desirable features for cell carriers and yeast selection for the production of 2G bioethanol; (ii) benefits and drawbacks of the different types of immobilization systems investigated to date, and (iii) final recommendations for the industry. This review article provides foundation knowledge that could serve as a platform for further application in the industry or research in the field of 2G biofuels.

Cell carriers and yeast selection for production of 2G bioethanol

Accurate selection of the immobilization technology and the material of the carrier is essential for any efficient cell-immobilization system. Operating costs, material stability, product quality, legality, and safety must be considered before using cell carriers.[41,42] Among the production systems that have been investigated, sodium alginate, polyvinyl alcohol (PVA) hydrogel, and lens-shaped particles (Lentikats®) seem to meet the above requirements and lead to improved ethanol yields or usability / reusability after long periods.[43,44] Research on yeast immobilization for cellulosic ethanol has been increasing over the last 10 years,[37,45,46] and future research should be geared towards developing resistant, economical, and abundant carriers to support their implementation in the biofuel industry. Optimal carrier requirements differ depending on the immobilized microorganism and the fermentation conditions but, generally, certain traits should be considered (Table 1).[41]

Before selecting a type of immobilization system, caution should be focused on the effects of the material over the yeast physiology to avoid metabolic modifications that, in turn, could affect the fermentation process and the product yield.[56] Some commonly observed effects are increase in stored polysaccharides, modified growth rates, lower by-product formation, activation of energy metabolism, increased substrate uptake and product yield, higher intracellular pH, changes in membrane permeability for protons, and abnormal enzyme activity (e.g. higher invertase activity).[42] High endurance has also been documented for immobilized yeast cells, which is thought to be because of the enhancement of production of carbohydrates, such as glycogen, along with other protective compounds.[57]

The selection of the yeast species and strains to immobilize depends on the cell adhesion properties and the conditions of the bioconversion process to be carried out. *Saccharomyces*

Table 1. Optimal cell-carrier requirements and examples of immobilization that exhibit the features.

Optimal carrier requirements	Main immobilization systems	Reference
Simple to manipulate	Auto-immobilization, immobilization on a support surface, mechanical containment behind a barrier	41,42,47
Sterilizable, reusable, and easy to recover	Mechanical containment behind a barrier	41
High cell mass-loading capacity, viability	Entrapment in a porous matrix	41,48
High surface-to-volume ratio, along with chemical groups enhancing cell-cell adhesion	Entrapment in a porous matrix, artificial inorganic	41,48–50
No harmful effect on yeast catalytic power	Auto-immobilization, mechanical containment behind a barrier, natural, artificial organic	41,47,51,52
Even and adjustable porosity (for exchange of nutrients and other substances with the media)	Entrapment in a porous matrix, artificial inorganic	48,50,53
Ease of optimal mass transfer	Auto-immobilization, immobilization on a support surface	42,47,48
Affordable and simple scale-up techniques	Entrapment in a porous matrix, immobilization on a support surface, natural	42,48,50,54
Chemical, mechanical, thermal, and biological stability	Entrapment in a porous matrix	48
Non-toxicity and no effect on the final product	Mechanical containment behind a barrier, natural, artificial organic	41,48,50,51
Suitable for different types of reactors	Entrapment in a porous matrix, artificial organic	55
Economical price	Entrapment in a porous matrix, immobilization on a support surface, natural	42,48,50

cerevisiae, *Kluyveromyces marxianus*, and *Pichia stipitis* are major species that have been studied in immobilized format to produce 2G bioethanol (Tables 2–4). *Saccharomyces cerevisiae* has been successfully immobilized in all types of immobilization: entrapment in a porous matrix, attachment on a support surface, and mechanical containment behind a barrier.[42] Further, its cell-to-cell adhesive property permits auto-immobilization such as flocs or biofilms.[45,58,74,75] *Saccharomyces cerevisiae* is frequently used for bioethanol production because it is naturally tolerant to ethanol and chemical inhibitors, it is easily genetically manipulated, and is high in ethanol yield. However, it inability to ferment pentoses and its low tolerance to high temperatures limit the yeast usefulness to the fermentation of lignocellulosic hydrolysates at mild temperatures.[76]

Kluyveromyces marxianus ferments a wide variety of sugars and has a high optimal growth temperature, which helps to lower contamination risks and to avoid expense for cooling systems.[77] However, low ethanol yields and excess sugar after fermentation have been reported because of unwanted by-product release (e.g. xylitol) and its strong Crabtree-negative nature.[78] *Kluyveromyces marxianus* has been proven to immobilize efficiently in biochar, an organic immobilization system, by either physical adsorption by electrostatic forces, natural cell entrapment onto a porous support, or covalent bonding between a membrane and the support. Kyriakou *et al.* (2019)[67] highlighted the ethanol productivity of 7.3 g Lh^{-1} by a biochar *K. marxianus*-based biocatalyst.

Pichia stipitis also has an inherent ability to ferment xylose and other sugars typically contained in lignocellulosic hydrolysates. It presents high ethanol yields and has an enzyme with an exo-1,4-cellobiohydrolase activity, which makes saccharification-fermentation integrated processes possible.[79,80] However, it requires specific fermentation conditions because it is sensitive to harsh conditions and assimilates part of the ethanol it produces.[81] *Pichia stipitis* has been efficiently co-immobilized along with *Trichoderma reesei* and *S. cerevisiae* in biofilm membranes. The ethanol productivity using this immobilization technology was almost twofold higher than when the same yeasts were used in suspension and supplemented with cellulases.[45] Further, *P. stipitis* cells have also been successfully entrapped in alginate beads.[61]

Comparison of the yeast immobilization systems for 2G bioethanol production

Immobilization methods depending on the yeast cell localization

Based on the physical localization and the nature of the microenvironment, immobilized cell systems can be arranged into four categories: auto-immobilization, entrapment in a

Table 2. Yeast auto-immobilization systems applied for second-generation bioethanol production. Should be noted that multispecies biofilm membrane is a hybrid immobilization system that gathers yeast cell auto-immobilization and mechanical containment behind a barrier.

Yeast immobilization carrier	Immobilized yeast	Raw material	Initial substrate concentration (g/L)	Fermentation condition	Ethanol production (g/L)		Ethanol productivity (g/Lh)		Reference
					In immobilized format	In non-immobilized format	In immobilized format	In non-immobilized format	
Multispecies biofilm membrane (N-O)	*Saccharomyces cerevisiae* and *Pichia stipitis*	Avicel	10*	Semi-continuous CBP, 28 °C, 240 h	3.5	-	0.02	-	45
		Avicel	17.5*	Batch CBP, 28 °C, 216h	7.2	-	0.04	-	
		Wheat straw slurry	17.5*	For the immobilized format: batch CBP, 28 °C, 144 h; for the non-immobilized format: batch SSCF supplemented with 15 FPU cellulase/g glucan, 28 °C, 150 rpm, 144 h	9.8	5.5	0.07	0.04	
		Washed pretreated wheat straw supplemented with xylose	17.5* and 22 xylose		9.1	6	0.06	0.04	
Self-flocculation (N)	*S. cerevisiae* KF-7	Diluted waste molasses	180	Continuous fermentation, 30 °C, 150 rpm, 35 days	80	-	6.6	-	58

CBP, consolidated bioprocessing; N, natural support; O, organic support; SSCF, simultaneous saccharification and co-fermentation.
*cellulose.

porous matrix, immobilization on a support surface, and mechanical containment behind a barrier (Fig. 2).

Auto-immobilization

Although auto-immobilization has been extensively used in other industries (e.g., winemaking or brewing), its application for 2G bioethanol production is rather limited. Some yeast strain cells can naturally aggregate by interactions with one another, forming several multi-cellular aggregations like biofilms or flocs. Adverse environmental conditions can trigger yeasts such as *S. cerevisiae* to adhere to other cells, which enhances the utilization of accessible resources of the medium, thus boosting its stress endurance and maximizing its lifetime.[41,82] This type of immobilization is directly influenced by the environment's physical, chemical, and biological factors. Auto-immobilization is directly related to the activity of a group of cell-wall glycoproteins called adhesines or flocculins, which are crucial in many inter-cell processes, like flocculation or fungal biofilm formation.[83,84] Although auto-immobilization occurs naturally, extra compounds like artificial flocculating agents or crosslinkers may be added to enhance the process. Some linking agents are polyelectrolytes, coupling agents by covalent bond formation, or inert powders.[41]

Multispecies biofilm membranes (MBM), a novel form of auto-immobilization system for 2G biofuel production, have been designed by Brethauer and Studer (2014)[45] (Table 2). This system involves two types of immobilization: immobilization on a support surface and auto-immobilization (biofilm). It is composed of a permeable membrane covered with a two-layered biofilm, consisting of a *T. reesei* filamentous fungus biofilm and a *S. cerevisiae* and *P. stipitis* yeast biofilm on top. The aerobic enzyme-producing fungus *T. reesei* grows directly on the oxygen permeable membrane and hydrolyzes the carbohydrate fraction of lignocellulosic biomass to reducing sugars. The hexoses are then fermented by *S. cerevisiae* and the pentoses by *P. stipitis* in the anaerobic parts of the reactor. Brethauer and Studer (2014)[45] applied MBM to perform CBP to obtain ethanol directly from acid-pretreated wheat straw, and compared it with simultaneous saccharification and co-alcoholic fermentation (SSCF) using a co-culture of *S. cerevisiae* and *P. stipitis* in non-immobilized formats combined with a cellulolytic cocktail (15 FPU/$g_{cellulose}$). Despite difficulties in controlling the microbial consortium activity and in maintaining optimal fermentation conditions for the microorganisms, the ethanol titers achieved in CBP (up to 10 g L^{-1}) were higher than those in SSCF (5 g L^{-1}) (Table 2). This result may be due to the immobilization-promoted enhancement of tolerance against lignocellulose-derived inhibitors, as has been shown for *S. cerevisiae*,[34] or to the

| *Biofuels, Bioprod. Bioref.* **15**:1549–1565 (2021); DOI: 10.1002/bbb.2250

Table 3. Systems of yeast entrapment in a porous matrix applied for second-generation bioethanol production.

Yeast immobilization carrier	Immobilized yeast	Raw material	Initial substrate concentration (g/L)	Fermentation condition	Ethanol production (g/L)		Ethanol productivity (g/Lh)		Reference
					In immobilized format	In non-immobilized format	In immobilized format	In non-immobilized format	
Agar-agar cubes (O)	*Saccharomyces cerevisiae* MTCC 174	Sugarcane bagasse enzymatic hydrolyzate	50	Batch fermentation, 30 °C, 72 h	9.4	-	0.26	-	59
	S. cerevisiae CTRI	Mahula flowers	350	Batch fermentation, 30 °C, 96 h	25.2	24.83	0.26	0.26	54
Alginate beads (O)	*Pachysolen tannophilus* MTCC 1077	Peels of pineapple Ananas cosmosus	51.7	Batch SSF supplemented with 5 FPU cellulase/g substrate, 50 °C for 24 h (saccharification) and 30 or 32 °C (*P. tannophilus* and *P. stipites*, respectively) for 96 h (fermentation)	10.5	-	0.15	-	60
	Pichia stipitis NCIM 3498				10.9	-	0.15	-	
	S. cerevisiae and *P. stipitis*	Wheat straw hydrolysate	30	Continuous fermentation, 30°C	10.42	-	9.8	-	61
	S. cerevisiae CTRI	Mahula flowers	350	Batch fermentation, 30 °C, 96 h	25.8	24.83	0.27	0.26	54
	S. cerevisiae	Carrot discards	89.8	Batch fermentation, 30 °C, 200 rpm, 4 h	24.5	-	7.17	-	55
	Candida shehatae NCL-3501	Rice straw autohydrolysate	23.1	Batch fermentation, 30 °C, 150 rpm, 120 h	11.55	10.39	0.24	0.22	62
		Rice straw acid hydrolysate	20		9.4	7.4	0.20	0.15	
	S. cerevisiae MTCC 174	Sugarcane bagasse enzymatic hydrolyzate	50	Batch fermentation, 30 °C, 72 h	11.8	-	0.32	-	59

Table 3. (Continued).

Yeast immobilization carrier	Immobilized yeast	Raw material	Initial substrate concentration (g/L)	Fermentation condition	Ethanol production (g/L)		Ethanol productivity (g/Lh)		Reference
					In immobilized format	In non-immobilized format	In immobilized format	In non-immobilized format	
Alginate beads (O)	Xylose-fermenting *Saccharomyces cerevisiae* T18	Undetoxified sugarcane bagasse hemicellulose hydrolysate	118	Batch fermentation, 35 °C, 150 rpm, 8 h	30	-	5.70	-	37
	S. cerevisiae Itaiquara baker's yeast with xylose isomerase	Crude sugarcane bagasse hemicellulosic hydrolysate	75.5	Continuous fermentation, 35 °C, 150 rpm, 24 h	23.88	-	1.80	-	63
		Detoxified sugarcane bagasse hemicellulosic hydrolysate	98.7		23.17	-	1.80	-	
	S. cerevisiae BY4743	Saccharified liquid of laccase delignified Aloe vera leaf rind	42.5	Continuous fermentation, 40 °C, 6 h	15.30	14.47	2.55	2.41	64
				Continuous fermentation in packed bed reactor, 40 °C for 6 h	16.50	14.47	2.75	2.41	
	S. cerevisiae YPH499 and *Pachysolen tannophilus* ATCC 32691	Pretreated cotton stalk lignocellulosic biomass	20.0	Batch SSCF, 30 °C, 150 rpm, 96 h	9.21	9.81	0.10	0.10	65
	S. cerevisiae InvSc 1 with the ability to ferment xylose	Lime-pretreated rice straw	92	Batch SSF, 30 °C, 50 rpm, 240 h	35	35	0.13	0.13	36
Alginate-chitosan capsules (O)	Genetically engineered *S. cerevisiae* T0936 with the ability to ferment xylose	Wheat straw	51.4	Batch SSFF with 10 FPU cellulase/g suspended solids, 50 °C, 500 rpm (saccharification); and 30 °C, 150 rpm, 96 h (fermentation)	37.1	-	0.38	-	66
				Batch SSF supplemented with 10 FPU cellulase/g suspended solids, 35 °C, 96 h	21.9	-	0.23	-	

Table 3. (Continued).

Yeast immobilization carrier	Immobilized yeast	Raw material	Initial substrate concentration (g/L)	Fermentation condition	Ethanol production (g/L)		Ethanol productivity (g/Lh)		Reference
					In immobilized format	In non-immobilized format	In immobilized format	In non-immobilized format	
Biochar produced from corks (O)	*S. cerevisiae*	Citrus peel waste hydrolysate	72	Batch fermentation, 37 °C, 15 h	51	42	3.9	3.2	51
Biochar produced from peanut shells (O)					46	42	3.5	3.2	
Biochar produced from pistachio shells (O)					63	32	7.8	4	
Biochar from non-biological origin (from recycled car tires) (O)	*S. cerevisiae*	Valencia orange peel hydrolyzates	90	Batch fermentation, 37 °C, 100 rpm, 10 h	60	55	6	5.5	67
	Kluyveromyces marxianus			Batch fermentation, 42 °C, 100 rpm, 10 h	56	50	5.6	5	
	Pichia kudriavzevii KVMP10				25	12.5	2.5	1.2	
Biochar from seagrass residue (O)	*S. cerevisiae*			Batch fermentation, 37 °C, 100 rpm, 10 h	52.5	55	5.2	5.5	
	K. marxianus			Batch fermentation, 42 °C, 100 rpm, 10 h	50	50	5	5	
	P. kudriavzevii KVMP10				12.5	12.5	1.2	1.2	
Biochar from vineyard prunings (O)	*S. cerevisiae*			Batch fermentation, 37 °C, 100 rpm, 10 h	72	55	7.2	5.5	67
	K. marxianus			Batch fermentation, 42 °C, 100 rpm, 10 h	73	50	7.3	5	
	P. kudriavzevii KVMP10				12.5	12.5	1.2	1.2	
κ-carrageenan (O)	*Saccharomyces cerevisiae* ATCC 24553	Pineapple cannery waste	82.3	Continuous fermentation, 30 °C, 87 days	28.5	24.5	42.8	3.8	68
Lentikat ® discs (I)	*S. cerevisiae*	Oilseed rape straw	60	Continuous fermentation, 30 °C,18 days	25.8	-	12.88	-	69
Luffa sponge discs (O)	*S. cerevisiae* CTCRI	Mahula flowers	89.7	Batch fermentation, 30 °C, 96 h	37.2	33.8	0.39	0.35	70

I, inorganic support; O, organic support; SSF, simultaneous saccharification and fermentation; SSCF, saccharification and co-fermentation SSFF, simultaneous saccharification, filtration.

Table 4. Systems for yeast immobilized on a support surface applied for second-generation bioethanol production.

Yeast immobilization carrier	Immobilized yeast	Raw material	Initial substrate concentration (g/L)	Fermentation condition	Ethanol production (g/L)		Ethanol productivity (g/Lh)		Reference
					In immobilized format	In non-immobilized format	In immobilized format	In non-immobilized format	
Cashew apple bagasse (N)	*Saccharomyces cerevisiae* Baker yeast	Cashew apple juice	70.1	Batch fermentation, 30 °C, 150 rpm, 6 h	36.91	38.57	6.15	4.29	71
Modified corn stalk (O)	*S. cerevisiae* CGMCC 2982	Concentrated food waste hydrolysates	202.6	Batch fermentation, 30 °C, 100 rpm, 74 h	87.91	82.23	1.83	1.37	72
				Continuous fermentation, 30 °C, 40 days	84.85	-	43.54	-	
Delignified cellulosic material from sawdust (O)	*S. cerevisiae* commercial baker's yeast	Molasses + residues of oil extraction process (OOMW) diluted with tap water (1/1 ratio)	140 (molasses) and 25 (OOMW)	Batch fermentation, 20 °C, 48 h	67.8	64.8	2.82	1.52	46
Reticular hollow sphere made of polyethylene and three layers of cotton fiber (O)	Angel active dry yeast TH-AADY	Expired rice	250	Batch fermentation, 30 °C, 48 h	98.63	94.76	2.05	1.97	73
Sugarcane bagasse (N)	*S. cerevisiae* MTCC 174	Sugarcane bagasse	50	Batch fermentation, 30 °C, 72 h	15.4	-	0.42	-	59

N, natural support; O, organic support.

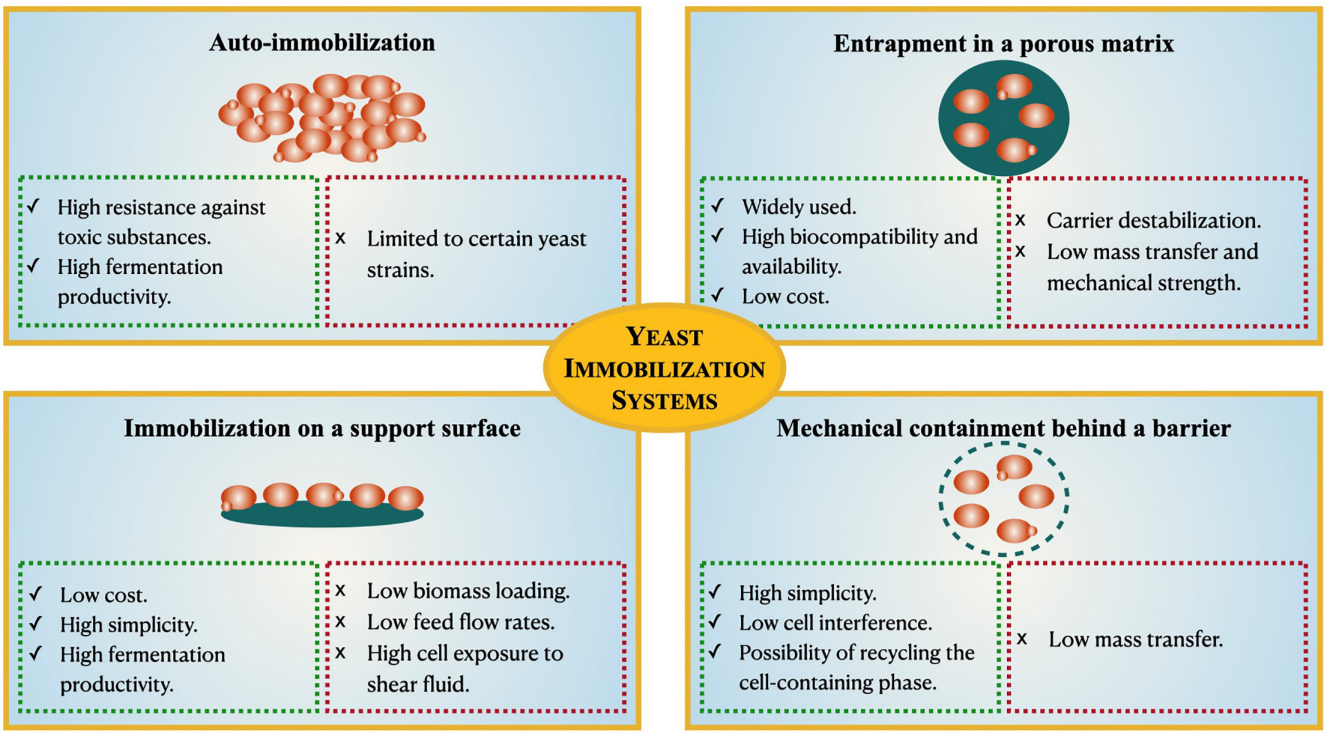

Figure 2. Immobilization methods depending on the yeast cell localization: advantages and disadvantages.

unique potential of the biofilm growth mode to facilitate an efficient substrate utilization and increased product yield.[85] It has also been suggested that mixed cultures avoid capacity loss and robustness loss of the biocatalyst.[86]

Another proposed example of auto-immobilization for 2G bioethanol production is the flocculation of the yeast strain *S. cerevisiae* KF-7, which is a very convenient approach for ethanol distilleries that use traditional tanks reactors.[58] Cell flocculation is commonly used in the brewery and sparkling wine industries, and it consists of the asexual, homotypic, and reversible aggregation of single-celled organisms in suspension to create a larger unit or aggregates called flocs.[42,87,88] Tang *et al.* (2010)[58] employed the flocculant *S. cerevisiae* KF-7 strain to ferment diluted waste molasses with a sugar concentration of 180 g L^{-1} in a two-stage continuous fermentation process. This resulted in achieving a maximum of 80 g L^{-1} of ethanol production and ethanol productivity of 6.6 g L^{-1}h. The authors state that the results with this method are much better than those obtained with traditional technologies, and they propose the use of *S. cerevisiae* KF-7 for ethanol distilleries. This strategy led to less contamination than conventional methods, but less immobilization was reported when the stirring rate was elevated. In our opinion, even if the results were achieved in the fermentation of a non-lignocellulosic substrate, the method is also applicable for 2G ethanol-producing distilleries. Yeast immobilization through flocculation lowers production costs and permits a more ecological process.[47] Based on the clear advantages of self-flocculation, this immobilization method deserves a more frequent application in the biofuel industry.

Entrapment in a porous matrix

Entrapment in a porous matrix is defined as the confinement of the yeast cells inside a carrier while allowing interaction with the medium (metabolism, mass transfer, nutrient exchange, etc.).[89] In this case, cell containment can be achieved through two different methods: by direct immobilization of the cells within the formation of the carrier or by releasing the cells into an already existing matrix. This method is the most investigated in the 2G biofuel production industry; however, some limitations need to be considered: carrier destabilization due to low pH values, diffusion of gases (like CO_2), and overgrowth of the microorganism.[32] The presence of certain substances like phosphates in the medium can also lead to weak carriers.[48] Other drawbacks of this system are severe mass transfer limitations, low mechanical strength, and large pore size.[53] Nonetheless, this type of technology brings some advantages such as biocompatibility, low cost, and high availability.[55] High densities of entrapped cells in the matrix can be reached, and, compared with surface immobilization, cells are less exposed to shear fluid.[41]

Several different entrapment methods, e.g. agar-agar cubes, alginate and alginate-chitosan capsules, biochar, κ-carrageenan, Lentikat® discs, and luffa sponge discs, have recently been described (Table 3). The method that has been investigated most is based on the use of calcium alginate (CA) beads as an entrapment system. The popularity of CA beads in alcoholic fermentation is mostly due to the ease of preparation and the non-requirement for severe operational conditions.[55] To obtain the beads, yeasts are mixed with a sodium alginate solution and dripped into a solution containing Ca^{2+} ions, where the resulting insoluble CA droplets form spheres containing the cells.[90] Several authors have used these gel matrices to produce ethanol from lignocellulosic material (Table 3). For instance, Ishola *et al.* (2015)[66] encapsulated a xylose-utilizing recombinant strain of *S. cerevisiae* in alginate-chitosan to facilitate simultaneous utilization of glucose and xylose during bioconversion of a slurry of acid-pretreated wheat straw. This approach resulted in a final ethanol concentration of 37.1 g L^{-1}, which corresponds to 90% of the theoretical yield. Although the incorporation of chitosan into the capsule matrix makes it stronger,[35] significant damage to the beads was reported. These injuries were attributed to shear stress caused by agitation during mixing with the solid particles in the simultaneous saccharification and fermentation (SSF) process.[66]

Agar-agar, κ-carrageenan, Lentikat® discs, and PVA are other examples of synthetic polymers used as yeast carriers in 2G bioethanol production. Agar-agar cubes consist of agar powder mixed with a sterile NaCl solution with cells added at around 30 °C to ensure rapid gelification and further solidification inside a mold. The solidified agar block is then cut into cubes of the desired size. This last step distinguishes agar-agar cubes from other gel carriers previously described. Singh *et al.* (2013)[59] employed this method in an evaluation of different matrices to immobilize *S. cerevisiae* MTCC 174 for fermenting different batches of enzymatic hydrolysate of alkali-pretreated sugarcane bagasse (Tables 3 and 4). Behera *et al.* (2010)[54] compared fermentations with either yeast immobilized in agar or suspended yeast cells and found higher ethanol production for the immobilized cells (25.2 g L^{-1}) than with free-cell format (24.8 g L^{-1}) when fermenting mahula flowers.

K-Carrageenan is another polymer that gelifies rapidly when potassium ions are added to the medium. As agar-agar cubes, κ-carrageenan requires mild conditions to immobilize without drastic temperature (~35 °C) and pressure changes that could negatively affect the yeasts' endurance and viability. Nigam (2000)[68] immobilized *S. cerevisiae* ATCC 24553 in κ-carrageenan packed in a tapered glass column reactor for ethanol production from pineapple cannery waste. The maximum ethanol volumetric productivity and production were 42.8 g L^{-1}h and 28.5 g L^{-1}, respectively. Compared to free yeast cells, the volumetric ethanol productivity of the immobilized cells was 11.5 times higher. Lentikat® discs, a further synthetic carrier originally used to immobilize bacteria for nitrogen removal,[91] have also been used to immobilize yeast cells for bioethanol production. This kind of carrier mixes yeast-cell suspension with liquid Lentikat® and sets them on sterile Petri dishes to dry.[49,69] Mathew *et al.* (2014)[69] evaluated the effect of different experimental conditions on bioethanol production in the fermentation of oilseed rape straw hydrolysates using *S. cerevisiae* cells immobilized in Lentikat® discs in a packed-bed column reactor and obtained 25.8 g L^{-1} ethanol in 18 days with a maximum volumetric productivity of 12.9 g L^{-1}h.

Another polymeric compound that has been proposed as an alternative candidate for industrial applications is PVA, which is prepared by dropping a PVA solution mixed with yeast cells into a buffer solution.[32] Recultivation of the original culture medium is necessary before use to recover the activity of the cells.[92] Even though we did not find any references that utilize PVA to immobilize yeast cells for 2G biofuel purposes, the method has been used for fermenting lignocellulosic hydrolysates with ethanologenic bacteria. Wirawan *et al.* (2012)[93] immobilized *Zymomonas mobilis* cells in CA and PVA to ferment acid-pretreated bagasse using an SSF and separate hydrolysis and fermentation (SHF) process achieving maximum ethanol concentration (6.2 g L^{-1}) and volumetric productivity (3.0 g L^{-1}h) with PVA in the SHF process. These results were higher when contrasted with those of bacteria immobilized in CA beads, which gave maximum ethanol concentration and productivity of 5.5 and 2.4 g L^{-1}h, respectively. High values were also reported when conducting SHF: 5.5 g L^{-1} at 1.3 g L^{-1}h when immobilized on PVA and 5.4 g L^{-1} at 1.3 g L^{-1}h when immobilizing on CA.

Another recently used method is biochar immobilization. Biochar constitutes a carbon-rich material produced via pyrolysis of biomass (Kyriakou *et al.*, 2020).[51] Some of the feedstocks used in biochar production are cork, peanut shells, pistachio shells, seagrass residue, vineyard prunings, and non-biological materials (from recycled car tires) (Table 3). For example, Kyriakou *et al.* (2019)[67] studied the use of biochars obtained from recycled car tires, olive kernels, seagrass residue, sewage sludge, and vineyard prunings for the immobilization of the yeasts *S. cerevisiae*, *K. marxianus*, and *Pichia kudriavzevii* KVMP10. The highest ethanol production levels were found in the fermentation of Valencia

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orange peel hydrolysates with *K. marxianus* immobilized on vineyard pruning biochar, where the concentration achieved ($73\,g\,L^{-1}$) was much higher than the value produced with cells in suspension ($50\,g\,L^{-1}$). According to Kyriakou *et al.* (2019, 2020),[51,67] biochar composition plays an important role in final bioethanol production.

Finally, although less frequently used, luffa sponge (*Luffa aegyptiaca*) discs have considerable potential for scaling up fermentation.[94] Luffa sponge discs are 2.5 cm diameter, 3–4 mm thick discs made of dried, tropical spongy fruit, where yeast cells are attached. Behera *et al.* (2011)[94] examined the ethanol production from mahula (*Madhuca longifolia*) flowers, a proven economic source for ethanol production, in submerged fermentation using whole cells of *S. cerevisiae* CTCRI immobilized in luffa sponge discs. Using that setup, the yeast cells remain physiologically active for up to four cycles of fermentation without a significant reduction in the amount of ethanol produced. After 96 h there was ethanol production of $37.2\,g\,L^{-1}$ and 8.96% higher for immobilized cells than the cells in suspension.

Immobilization on a support surface

This type of immobilization, also known as adsorption, is popular because of its simplicity, low cost, and high productivity. It consists of binding yeast cells to the carrier surface by electrostatic forces such as covalent bonds, ionic bonds, hydrogen bridges, or Van der Waals forces.[48] Adsorption can be accomplished by the yeast itself or by artificial induction using linking agents. In continuous ethanol production, adsorption is often accomplished by circulating a highly concentrated suspension of yeast cells through the bioreactor for several hours. To develop natural adsorption, several factors must be taken into consideration: properties of the support, the surrounding conditions, and the capability of the yeast strain to attach to the carrier.[41] Nonetheless, absorbed-cell systems are limited as biomass loading and feed flow rates are low compared with other immobilization strategies, i.e. entrapped-cell systems. This is because the number of cells that can be absorbed on the carrier is limited by its surface area.[48] Despite shortcomings, the low cost of the materials and the simplicity of the process have pushed forward the utilization of this method.[42]

Different types of carriers are used as support surfaces. Some examples are natural carriers like cashew apple bagasse, delignified cellulosic material (DCM), cotton fiber, or sugarcane bagasse. DCM, which is a delignified, washed and dewatered lignocellulosic material, is the main carrier used for absorption. Nikolaou and Kourkoutas (2017)[46] immobilized commercial baker's yeast on DCM to ferment a blend of olive oil mill wastewaters and molasses in two batches for 48 h. The fermentations resulted in a higher ethanol concentration ($67.8\,g\,L^{-1}$) than was achieved with free cells ($64.8\,g\,L^{-1}$). Sugarcane bagasse is another natural carrier of interest. Singh *et al.* (2013)[59] conducted a comparative study on ethanol production from enzymatic hydrolysate of alkali-pretreated sugarcane bagasse ($50\,g\,L^{-1}$ initial sugar concentration) using immobilized *S. cerevisiae* MTCC 174 on three different matrices, namely sugarcane bagasse, Ca-alginate, and agar-agar. The highest ethanol concentration ($15.4\,g\,L^{-1}$) and volumetric productivity ($0.4\,g\,L^{-1}$ h) were obtained when using sugarcane bagasse as support. Immobilization on bagasse allowed up to ten fermentation cycles to be run, whereas only four cycles were possible when agar-agar and CA were the carriers of choice. The utilization of cashew bagasse as a *S. cerevisiae* cell carrier has also been investigated, and stable fermentation performance with high final ethanol concentration ($36.9\,g\,L^{-1}$) and ethanol productivity ($6.2\,g\,L^{-1}$h) between the third and the tenth fermentation cycles has been reported.[71]

Mechanical containment behind a barrier

Mechanical containment behind a barrier normally consists of holding yeast cells behind membranes with small pore sizes. It is mainly used when the final product requires a minimal transfer of compounds and / or a minimum number of free cells.[70] The barrier assembled around the immobilized cells is commonly a polymeric membrane but other materials, such as ceramic or silicone rubber, can also be used.[41]

One of the main facts to consider when using this kind of methodology is the mass transfer limitations, which are determined by the pore size of the barrier, its structure, or even its affinity to water. When using preformed membranes, no extra conditions are required, and the interference for the immobilized cells is minimized.[41] Entrapment also allows for a two-phase system where substrates or products are partitioned separately, and avoids unwanted by-products. A strength of this methodology is the possibility of recycling the cell-containing phase, which is a challenging task with other immobilization strategies.

From a literature survey, we could only find the MBM system applied to CBP to obtain ethanol directly from acid-pretreated wheat straw, previously discussed.

Immobilization methods depending on the chemical composition of the carrier

Based on their origin, the support materials used in immobilization systems can be classified as natural and artificial materials (Fig. 3).

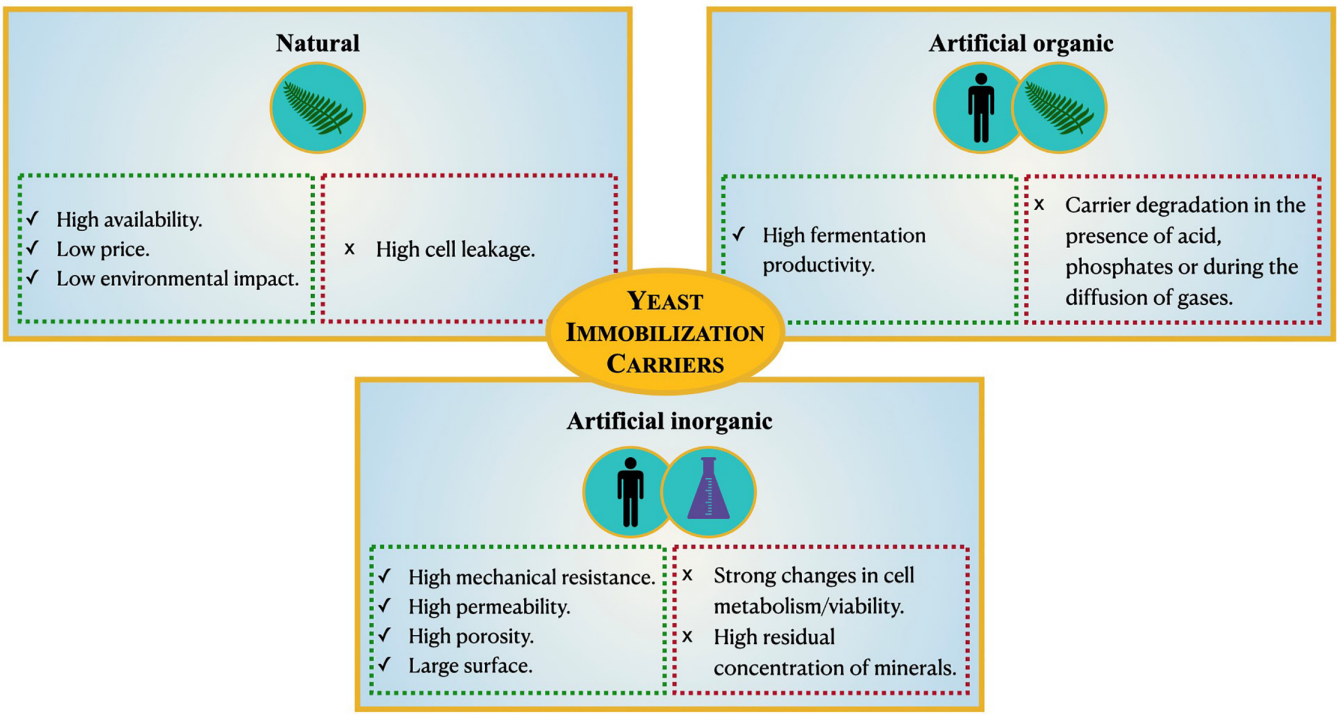

Figure 3. Immobilization methods depending on the chemical composition of the carrier: advantages and disadvantages.

Natural carriers

Natural carriers are usually produced directly from nature.[95] Because of the abundance, low price, and rather good purity of natural materials, their use is advantageous for immobilization methodologies.[48,50] Other advantages of natural materials is that they exert low impact on the environment, allow for efficient fermentation processes,[52] and increased yeast resistance to environmental stress.[51] Some examples of natural carriers investigated for the production of 2G bioethanol are sugarcane bagasse and cashew apple bagasse, which were already mentioned in previous sections (Tables 2 and 4). Many natural supports are waste materials from agro-industrial processes associated with major environmental issues. For this reason, natural carriers used to produce high added-value products, such as bioethanol, are expected to result in sustainable green development and simultaneously to resolve disposal-related problems.

Even though natural carriers bring up several benefits, some systems have an inherent flaw where yeast cells release from the support. These problems can be partially prevented by coating the immobilization system with a gel.[96]

Artificial carriers

Artificial carriers can be organic or inorganic. Despite their composition, organic supports are either synthetically made (e.g., plastic materials) or obtained from natural sources by complicated techniques (e.g., polymeric hydrogels). The carbonaceous composition of some organic supports affects nutrient availability and product release, and that influences the metabolic behavior of immobilized cells and, consequently, alcoholic fermentation.[51] Examples of organic carriers are biochar, cellulose, chitosan, and polymeric hydrogels like alginate (Tables 2–4).

Inorganic supports are made of materials like ceramics, glass or polyurethane foam.[42] They are abundant and display high mechanical resistance, adequate permeability, capacity, porosity, and a large surface.[48,50] Although inorganic supports can enhance fermentation productivity, they can produce high residual concentration of minerals in the final product and induce strong changes in the metabolism and viability of the immobilized cells. However, their use is promising for distillates or bioethanol production (Table 3). Artificial carriers may also improve some aspects of the 2G biofuel production process, such as high immobilization capacity, material flexibility, endurance, and viability of immobilized cells.[91]

Comparison of the immobilization systems investigated

Entrapment in a porous matrix, and more specifically, in CA, is the most frequently investigated system for 2G bioethanol production. CA features make it attractive for 2G bioethanol

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production;[32,48,55] however, in terms of improvement of bioethanol production and productivity versus free yeasts, CA beads are outcompeted by other immobilization systems (Tables 2–4).

Although high sugar-to-ethanol conversion rates were achieved by immobilizing a xylose-utilizing *S. cerevisiae* strain in alginate-chitosan capsules,[66] other authors reported low bioethanol yield and productivity when using alginate as the carrier.[54,62] This may be related to limits on mass transfer reactions, which hinder metabolite diffusion through the gel and decelerate the production and excretion of ethanol.[97] When comparing alginate with other matrices under the same fermentative conditions, the sugar cane matrix was found to overtake alginate beads in terms of ethanol productivity and the number of fermentation cycles.[59]

Cell carriers like biochar or MBM have been shown to improve ethanol yield and productivity versus free yeasts (Tables 2 and 3). The high ethanol production using biochar may be attributed to the positive effects of the carbonaceous structure on biofilm formation, buffering capacity, and nutrient adsorption.[67,98] In the case of MBM where the microorganisms are arranged in consortia, synergies may exist that can be translated to the increase of substrate utilization and enhancement of fermentation.[99] Further, biofilm growth mode has been associated with increased microbial resistance against toxic substances, reduced cell biomass production, and higher productivity.[85]

Biochar, besides improving ethanol production and productivity, offers other advantages over the alginate beads. Considering that biochar is generated from biowaste, its utilization enables the integration of thermal and biological methods targeting the manufacture of high added-value commodities and lowering the environmental impact.[100] The combination of 2G ethanol production with pyrolysis in biorefineries can allow energy gains and contribute to biowaste reduction.[101,102]

Conclusions

Yeast immobilization is a convenient technology to be implemented in the fermentation step of the 2G ethanol industry. The effectiveness of yeast immobilization has been demonstrated in laboratory-scale experiments but its massive industrial utilization is still far from being a reality. Alginate beads are the most investigated immobilization system in 2G bioethanol research, but the promising features of other systems, such as biochar or multispecies biofilm membranes can bring increased industrial potential.

Research should be directed to the identification of the optimal combination of immobilization technologies, lignocellulosic substrates, and bioconversion processes, to attain an efficient implementation in biorefineries. In this way, it will be possible to introduce yeast immobilization-based innovations and exploit their full potential in the 2G ethanol industry.

Acknowledgements

Kind help from Minami Ogawa for the translation of the article is gratefully acknowledged.

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