Bioresource Technology

Enzyme catalysis with artificial membranes towards process intensification in biorefinery- A review

--Manuscript Draft--

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Abstract:	In this review, for the first time, the conjugation of the major types of enzymes used in biorefineries and the membrane processes to develop different configurations of MBRs, was analyzed for the production of biofuels, phytotherapics, food ingredients. In particular, the aim is to critically review all the works related to the application of MBR in biorefinery, highlighting the advantages and the main drawbacks which can interfere with the development of this system at industrial scale. Alternatives strategies to overcome main limits will be also described in the different application fields, such as the use of biofunctionalized magnetic nanoparticles associated with membrane processes for enzyme re-use and membrane cleaning or the membrane fouling control by the use of integrated membrane process associated with MBR.

Subject: Revised review submission for publication in Bioresource technology

Rende, April 2021

Dear Editor,

we are greatly interested to submit a revised version an original review to Bioresource Technology, titled "Enzyme catalysis with artificial membranes towards process intensification in biorefinery".

We sincerely hope we were able to fully address the concerns of the reviewers and that, after revisions, the manuscript can reach the level expected for publication. We are grateful to the Reviewers for the opportunity they give us to enhance the quality of our work. As attached files you will find a detailed answer to referee comments and the two requested versions of the revised manuscript (with and without highlighted revisions).

As requested in the journal submission form I also declare that:

(1) the subject Classification is: "biomass and feedstocks utilization: bioconversion of agro-industrial residues"

(2) that all the authors agree for the submission to BITE

(3) that the review submitted is an original work of all the authors

(4) that our manuscript is an original work and it has not been previously published. The article is currently not under consideration for publication elsewhere.

In the following and in the "answer to referees comments" you will find the answer to editor-in-chief comments from last revision.

Editor in chief comments:
Page length can be maximum 50.
Answer: Following the editor-in-chief last revisions, the review was reduced to 50 pages (including references, tables and figures). In order to reach this aim paragraph 1.1 was removed since too general. Fig. 4 was also removed explaining the meaning in the text and Table 4 since also too general.

Conclusion can be maximum 100 words.
Answer: Conclusions was reduced to 100 words

Change title to Enzyme catalysis with artificial membranes towards process intensification in biorefinery - A review
Answer: Done ! Thank you

Thank you in advance for your cooperation.

Sincerely your,

Rosalinda Mazzei

(Corresponding Author)

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The pages and lines indicated in the answer to referee comments are referred to pages and lines to the revised version without highlights

Editor in chief comments:
Page length can be maximum 50.
Answer: Following the editor-in-chief last revisions, the review was reduced to 50 pages (including references, tables and figures). In order to reach this aim paragraph 1.1 was removed since too general. Fig. 4 was also removed explaining the meaning in the text and Table 4 since also too general.

Conclusion can be maximum 100 words.
Answer: Conclusions was reduced to 100 words

Change title to Enzyme catalysis with artificial membranes towards process intensification in biorefinery - A review
Answer: Done ! Thank you

Reviewers' comments:

Reviewer #1: This review describes in details the use of membrane bioreactors for process intensification in biorefinery. author describes literature in details. However, it has unnecessarily included general contents on biorfinery, which is not needed. Please delete all general details so that your paper becomes highly focused on the topic.
some comments/suggestions are as follows

1. I suggest to change the title of the article. while reading the article I confused between cell membrane and artificial membranes/bioreactors. Currently, it sounds quite unscientific and does not give what is there in the paper.
Answer: The title was modified as requested in "Enzyme catalysis with artificial membranes towards process intensification in biorefinery"

2. In biorefinery the pretreatment process is not same. These membrane bioreactors works only if the all polymers degrades e.g. if pretreatment process in acid based then lignin remains as it is so the enzyme seperation become difficult. The author did not discussed limitaitons of each membrane seperation process.
Answer: pre-treatment table was removed since too general for another reviewer

3. the future perspective is missing, may be added.
Answer: a new paragraph was now present before the conclusions, in which challenges and future perspective were reported, the title of the new paragraph is : "Challenges and future perspective on the use of MBR in biorefinery"

4. page 17: The attachment of enzyme to the cellulose particle....., I did not understand this?
Answer: in a free enzyme MBR, adsorption of enzyme cellulase onto the substrate cellulose is a big challenge. In this example, authors used this challenge as a strategy to retain the small molecular weight enzyme by high molecular weight membrane (0.6 um). To better clarify, the sentence page 14 line 337 is modified as:
"For instance, the natural tendency of enzyme to be adsorbed by cellulose, often a concern for enzyme efficiency loss, was taken as an advantage in order to retain the enzyme in 0.6 µm MF equipped submerged MBR for cellulose hydrolysis. While this system requires significant pre-holding time in order to ensure sufficient adsorption, the loss of enzyme is still unavoidable."

5. Remove Tables 1, 2, Fig 1, 2, by giving details in text only.

Answer: As suggested Tables 1, 2, Fig.1, Fig. 2 were removed and details were reported in the text

6. Sec 1.1 should be made very brief as part of Sec 1.2.
Answer: in order to follow journal rules (50 pages including references, figures and table) Section 1.1 was removed since too general.

7. Tables 4, 6 and 8 need newer refs of 2019-2020-2021.
Answer: newer references were now present in the revised manuscript in the mentioned tables except for pectinase and MBR, in this last field any new recent publication is reported in high impact factor journal in recent years:

Lim, S.Y., Ghazali, N.F. 2020a. Product Removal Strategy and Fouling Mechanism for Cellulose Hydrolysis in Enzymatic Membrane Reactor. Waste and Biomass Valorization, 11, 5575-5590.

Lim, S.Y., Ghazali, N.F. 2020b. Cellulose hydrolysis in an enzymatic membrane reactor: Fouling mechanism. IOP Conference Series: Materials Science and Engineering, 736, 022071.

Su Z., Luo J., Li X., Pinelo M., Enzyme membrane reactors for production of oligosaccharides: A review on the interdependence between enzyme reaction and membrane separation, Separation and Purification Technology, 243, 15 July 2020, 116840

Sokač T., Gojun M., Tušek A. J., Šalić A., Zelić B., Purification of biodiesel produced by lipase catalysed transesterification by ultrafiltration: Selection of membranes and analysis of membrane blocking mechanisms, Renewable Energy, 159, 2020, 642-651

Kumar, R. Pal, P., Lipase immobilized graphene oxide biocatalyst assisted enzymatic transesterification of Pongamia pinnata, 211, 2021, Fuel Processing Technology, 106577

8. Place each table/figure on separate page and put the end of the text.
Answer: all the figures and tables were placed in a separate page at the end of the text

9. Place text in double space.
Answer: DONE

10. Number the refs in the list.

Answer: the references were numbered in the reference list, following journal rules

Reviewer #2: The manuscript entitled 'Enzymes combined with membranes in biorefineries' puts forth a review on the integration of enzymes and membranes in the membrane bioreactors (MBRs) towards process intensification in biorefinery. Though the Authors have sufficiently discussed on the covered topics, the manuscript suffers from the following gaps which are essential to be addressed before its acceptance.

*Title is confusing.
Answer: Thank you for the kind suggestion! The title was modified as requested as "Enzyme catalysis with artificial membranes towards process intensification in biorefinery"

*All Highlights have to be more specific revealing the novelty of this review manuscript. Besides, it is essential that the highlights are presented in acceptable English. As highlight 4 'MBRs promote increasing in yields and conversion', does not standalone, it needs to be reframed precisely.

Answer: All the highlights have been corrected as follow:
~~1) Membrane processe and biocatalysis promote process intensification in biorefinery~~
1) Enzymes combined with artificial membranes in biorefinery promote process intensification
~~2) Membrane bioreactors (MBR) in biorefinery promote enzymes re-use and stability~~
2) The use of MBRs in biorefinery permit enzymes re-use and increased stability
~~3) MBRs in biorefinery promote removal of enzyme inhibitors and continuous operation~~
3) The use of MBRs promote removal of enzyme inhibitors and continuous operation
~~4) MBRs promote in yields and conversion~~
4) The use of MBR in biofuels, phytotherapics and food ingredients production was analyzed

*Title needs to be revised and made more crisp and intriguing to the readers. Besides, the core of investigation has to reflect in the title along with its applicability.
Answer: the title was changed as suggested "Enzyme catalysis with artificial membranes towards process intensification in biorefinery"

*Abstract-Authors need to improve the abstract by clearly stating the main aim of the review manuscript and the methodology adopted in strategizing the biocatalysts and membrane systems for the production of biofuels, phytotherapics and food ingredients along with the major conclusions drawn. Besides, the novelty of the present manuscript has to be emphasized in the abstract to reveal the originality of this work.

Answer: following referee suggestion, the abstract was modified following the referee suggestion and it was significantly reduced in order to follow journal rules.

*Introduction- The entire information provided in section 1 is well known and already published. This section can be shortened, besides, it is suggested to discuss how this manuscript is different from the available literature. What progress against the most recent and similar state-of-the-art studies was made in this research?

Answer: Section 1 was shortened (section 1.1 removed since too general) and the aim of the review was added in the introduction, highlighting that this is the first example in which this technology was reviewed in biorefinery.

*Table 1-It is suggested to either retain 'x' or '✓' for milling and also spell-check the terminology used.
Answer: the first referee suggested to cancel the table so it was eliminated

*Table 2 represents conventional information with no novel inputs. It is suggested to omit this table.
Answer: DONE

*Figure 1 and 2- Similarly these two figures are also not sharing any new information.
Answer: the two figures were removed!

Figure 3 can be revised to make it more scientific and attractive.
Answer: DONE

*Table 3- Authors are suggested to elaborate the information provided in the table in terms of applicability, major results and references.
Answer: A column was added to the mentioned table reporting the references and a new part explaining applicability and major results is now present from page 5 line 107 to page 6. We have tried to concisely cover the whole membrane processes and biocatalysis configuration, highlighting the examples most close to the topic, published on high impact factor journals.

*Table 5- Only limited studies have been cited in the table. It is advised to discuss those studies in the respective text and omit the table.
Answer: ok table five was deleted and the text on page 12 line 278 is modified as "Yet, the obtained product concentration in many of the studies is considerably low (0.2-20 g/L, see Table 52) (Gebreyohannes et al., 2018; Lim & Ghazali, 2020; Lozano et al., 2014; Zhang et al., 2011). Since the desired concentration for subsequent fermentation to ethanol, falls between 150 to 250 g/L glucose (Malmali et al., 2015), a significant energy is consumed in pre-concentration. Increasing the substrate concentration specially when using high MWCO membrane can be one strategy to achieve a higher product concentration (Malmali et al., 2015). In all these discussions, it was difficult to elucidate the contribution of the enzyme, as the type, amount and units of the enzymes used were different."

*Section 2.1.3- 'Biocatalytic membrane reactors in cellulase hydrolysis' is advised to be changed to 'Biocatalytic membrane reactors in cellulose hydrolysis'.
Answer: Done

*Section 2.3- 'Xilanase and MBR in biorefineries' has to be changed to 'Xylanase and MBR in biorefineries'.
Answer: Done

*Conclusions- It is suggested to rewrite the conclusions by providing data of key findings, novelty and applicability. Also follow the word count as stated in the author guidelines.
Answer: A new paragraph was introduced before the conclusion called: "Challenges and future perspective on the use of MBR in biorefinery " , in which the more important strategies discussed in the review were highlighted together with the main limits which need to be overcome in order to apply this technology on industrial scale. Conclusion section was modified and reduced according to journal rules.

*Authors are suggested to consider updating the manuscript by rigorously referring to the most recent and relevant references that have been published in high impact factor journals.
Answer: the manuscript is now updated with recent references as indicated in the answer to reviewer no 1. The research of new articles was carried out using both Scopus and WoS and different keywords, which include: membrane bioreactor and enzyme, pectinase and membrane bioreactor, lipase and membrane bioreactor, b-glucosidase and membrane bioreactor, cellulase and membrane bioreactor, xylanase and membrane bioreactor, enzyme membrane reactor, membrane bioreactor and biorefinery etc.. Beside high impact factor journal were also checked with the same keywords previously mentioned.

*A new and interesting direction to this review can be given by including a separate section on challenges in maintaining biocatalyst and membrane stability and cost constraints in real-field applicability. Also details on the way forward to overcome these challenges are advised to be discussed.
Answer: as previous highlighted future perspective, challenges and new solutions are now included in the revised manuscript in a new paragraph called "Challenges and future perspective on the use of MBR in

biorefinery". For what concerns the cost analysis the technology is at an emerging state of development in biorefinery, so these studies are not yet carried out. Besides, different new parts were also added in the abstract and in the introduction, which takes into account the novelty of the contribution given by this review and the several strategies to improve the main problems related on the development of these systems on industrial scale.

*Section 2.1 is very lengthy. Authors are suggested to make it more to the point and crisp.
Answer: DONE

*Author's need to check the reference style and maintain uniform format with respect to issue numbers, journal abbreviations and En Dash used amidst page numbers.
Answer: DONE

*Overall English grammar and framing of sentences needs to be revised to improve readability and match the journal standard. The manuscript needs language correction and spell-checks.
Answer: DONE

Reviewer #3: This review surveys the literature on the use of membrane bioreactors for enzymatic conversion of biomass feedstocks. Such bioreactor systems have the potential to overcome the operational and cost limitations of conventional batch or continuous bioreactors. Overall, the authors have succeeded in delivering a large body of information, particularly via the extensive tables and (mostly) well-rendered figures.

The authors have made the reviewer's task more difficult by not providing line numbers in the manuscript and by not indenting or separating successive paragraphs. Please correct these formatting deficiencies in any revision of the manuscript.

Answer: DONE

Specific comments:

Abstract- 2 nd ,3 rd (e.g. wood grass, leaves, microalgae, etc.) and 4 rd..... .....2nd, 3rd, ... and 4th....

Answer: This part was removed from abstract in order to respect journal rules about abstract length

P1, Introduction, paragraph 4, and P2, paragraph 1: The authors have chosen to lead off their review with a description of different "generations" of biomass bioconversion technologies, but this strategy is a little bit diversionary. The recent coinage of the terms "third generation biofuels" and "fourth generation biofuels" is unfortunate, especially since there is no evidence that even the so-called second-generation biofuels will ever be practically realized. Shouldn't one generation logically follow another? Second generation biofuels based on carbohydrate polymers logically follow first generation biofuels based on the component sugars of carbohydrate biopolymers. How do "third generation" biofuels arise from second generation biofuels? To this reviewer they do not, they are merely a separate, unrelated platform. Do we really want to get into a situation where every different platform gets to claim its own "generation" of biofuels? If so, we will soon be talking about tenth, or twentieth-generation biofuels! The reviewer suggests instead that the authors frame the discussion into two general types of bioconversions, namely polysaccharide conversions and lipid conversions.
Answer: We agree with the referee and we referred in the revised manuscript just to biorefineries generations as reported in the current literature, removing 4th generation. Unfortunately, the two types of suggested bioconversions cannot include all the applications treated in this review. For example the hydrolysis of oleuropein in the paragraph "β-glucosidase and membrane process in biorefinery". Oleuropein (the substrate) is not a polysaccharide and is not a lipid is a biophenol! For this reason the introduction was rewrote, taking into account the main finding and novelty of the review and referring just to second generation biomass.

P3, last line: The insolubility of cellulose is not conferred by its crystalline structure, but by its enormous chain length and by the additivity of many (rather weak) hydrogen bonds that permit aggregation into fibers. Amorphous cellulose, despite its lack of crystalline structure, no more water soluble than is crystalline cellulose.

Answer: thank you for the suggestion, however in order to follow jurnal rules (50 pages including references, tables and figures we have removed the paragraph 1.1 since too general as also suggested by the editor-in-chief.

P4, paragraph 3: The vague statement regarding the "very low content of lignin" in herbaceous plants needs clarification. How low? Many herbaceous plants contain substantial amounts of lignin (for example, approaching 10% of DM in lucerne).

Answer: see previous comment

P4, Table 2: This table is superfluous and does not really add to the review. It would suffice to simply state in the text that economical cellulosic biomass conversion will probably require some form of pretreatment, and many such pretreatments have been extensively studied.

Answer: Table 2 was removed together with paragraph 1.1

P5, last paragraph: The first few sentences are confusing and inaccurate, as they imply glucose as the sole hydrolytic product. The sentences should be modified to more effectively introduce the later sentences in the paragraph, which do a good job of explaining the hydrolytic products of the different classes of cellulases. The author should also mention that cellulases may be either complexed and cell associated (as in cellulosomes) or noncomplexed and extracellular.

Answer: paragraph 1.1 was removed since too general for the editor-in-chief

P6, paragraph 1: Most readers will probably be rather unfamiliar with these monooxygenases, so the authors should provide a literature citation that describes them more fully.

Answer: paragraph 1.1 was removed since too general for the editor-in-chief

P9, Figure 3. This figure is useful, but it would help to add some detail to the legend, for example by stating that in the BMR the biocatalyst is immobilized on or in the membrane. This information is in the text, but it would help the reader to have this reinforced when presented with the figure.

Answer: A new Fig. is now present in which the different configuration were highlighted, also the figure caption was rewrote in which the difference between BMR and MBR was also reported.

P10, paragraph 1, last sentence: In what way are they more beneficial? Higher throughput? Less fouling? More complete separation?

Answer: the sentence pag. 7 line 167 “which can be more beneficial from operational point of view, are used.” was changed in “which can be more beneficial in terms of membrane fouling control”

P10, Section 2.1.1, paragraph 1, L1-3: This statement should be qualified. The expectation is for 100% hydrolysis of the cellulose component of biomass, but because cellulose is only half or less of the biomass weight, the expected hydrolysis is reduced accordingly.

Answer: we agree with the referee, the sentence was wrong and “lignocellulosic biomass” was changed in “cellulose”

P10, Section 2.1.1, paragraph 3: The 19% conversion lacks context. What was the initial concentration of cellulose? One could probably obtain near 100% conversion if the substrate concentration was sufficiently small. (Also P11, L1; P11, paragraph 1)

Answer: noted and the substrate concentration is now added to the discussion as (pag.8 line 193): “Authors achieved 19% degree of conversion after 3 days, for a reasonable feed concentration of 25 g/L.”. Besides in Table 2 a column related to feed concentration is introduced.

P10, Section 2.1.1, paragraph 4: Do the authors mean that 95% of the cellulase (rather than cellulose) was retained?

Answer: YES, corrected at pag 8 line 197, thank you!

P11, paragraph 4, line 11: The phrase "a constant reaction rate over time" suggests that the system was enzyme-limited. Are such reaction conditions the most beneficial for optimizing the economics of cellulosic biorefineries, i.e., is it motivated by the high cost of enzyme?

Answer: yes! Because, if we increase the mass of enzyme by increasing the particle concentration the system will be mass transfer limited due to particle aggregation and the subsequent loss of biocatalytic efficiency.
To better clarify this concept the following sentence is added to the revised manuscript on page 10 line 241: "Use of biofunctionalized nanoparticles have the inherent issue of nanoparticle aggregation at high concentration. Hence, designing the system under reaction rate limited regime can prevent mass transfer resistance due to particle aggregation and the subsequent loss of biocatalytic efficiency."

Table 4: This is a useful table, but it's a little hard to draw informative comparisons among the different reports. For example, the per cent conversion of substrate varies substantially across studies, but this could simply reflect different initial concentrations of biomass. It might be more useful to include a separate column of substrate concentrations.
Answer: A separate column about substrate concentration was included in the revised table 2

P15, paragraph 1, L14: What is "amino acid pretreated corn stover"? What amino acids are used in pretreatment? Do the authors just mean acid-pretreated instead?
Answer: here the corn stover was incubated in 15 wt.% aqueous ammonia at a ratio of 1 g solid per 8 mL liquid at 60°C for 16 h, without agitation.
To better clarify the sentence it is modified as (pag 12 line 273): " For instance, a corn stover pre-treated by soaking in 15 wt% aqueous ammonia incubated with a cellulase loading of 60 FPU per initial cellulose was used to compare the performance difference among batch, continuously fed and intermittently fed MBR. Intermittent addition of 5 g/L cellulose every 8 h, gave a total glucose of 1.94 and 1.88 times higher than batch reactor without MBR and continuously fed MBR, respectively."

P15, paragraph 2: It would help here if the authors gave a brief description of EUF. What is its underlying principle? Does the applied current aid in filtration per se, or does it just decrease the extent of membrane fouling?
Answer: electro ultrafiltration is a principle applied to prevent membrane fouling via an applied voltage difference across the membrane. Depending on the surface charge of the foulant, an opposite charge electrodes are placed at the opposite side of the membrane in order to achieve electro static repulsion of membrane foulants.
The following remark is added on page 12 line 289 : "EUF is a method, where a differential electric field is applied across the membrane to achieve electrostatic repulsion of membrane foulants."

P16, Table 5: What is meant here by "product"? Is it specifically glucose, or does it include all soluble sugars (e.g., including oligosaccharides)?
Answer: Yes both glucose and oligosaccharides. Another referee suggested to remove the table and include the data in the text so it was removed and better clarified in the main text.

P18, L2-3: Are the authors referring here to enzymes in general, or more specifically to cellulases?
Answer: if you are referring to the sentence of immobilized enzyme, the answer is yes, it is referred to enzymes in general! Since in the references reported (Di Cosimo et al.) an overview on the industrial application of immobilized enzymes is reported. The sentence was removed since too general

P18, paragraph 2, L9: The units here seem inappropriate for a solids loading rate.
Answer: units are now amended and reported as "3-6 g/h" (pag 16 line 357)

P18, Section 2.2, L2: Perhaps "accelerating" rather than "determining".

Answer: Yes, it was modified

P19, Figure 4: The figure is useful, but the legend should identify PMWW as olive mill wastewater, and indicate that oleuropein is the aglycone. Also, the "NI" in Fig.4A should be "IN". Finally, it appears in Fig. 4B that the oleuropein appears in both the aqueous and organic phases. Does this mean that it some of it is extracted, and if so, does this mean it is lost without being converted to more of the aglycone?
Answer: Fig.4 and 5 (now Figure 2 and 3) and their captions were modified as suggested. For what concerns old Fig. 4 yes the conversion in the mentioned BMR was not complete, but it was optimized in the following ones (see Mazzei et al 2020), so unconverted oleuropein remained in the aqueous phase. The oleuropein aglycone is the product of the oleuropein hydrolyisis and it is present just in the organic phase. As reported on page 17 line 403 e "the glycosidic substrate is oleuropein while the product of hydrolysis is oleuropein aglycone".
In the caption of old Fig. 4 and 5 the following sentence was added: ". OA: oleuropein aglycone (product of oleuropein hydrolysis by β-glucosidase action)"

P21, Fig.5: It is useful that these panels are grouped together to allow comparison of the processes. But panels A and D, because of the small text size and its light color are extremely difficult to read. Also, legend should define "OLA" that appears in panel D.
Answer: The figures were now present in the revised manuscript in bigger size and with higher resolution, OLA was changed in OA because it means oleuropein aglycone, see previous answer and all the abbreviations were reported in figure caption.

P22, paragraph 2: Be more specific here to indicate that xylan has a tendency to form gel-like aggregates that can contribute to fouling, and that this behaviour also complicates pumping or circulating of xylan polymers.
Answer: the sentence at pag 18 line 431 was modified as following: "However, it must be considered that the substrate tends to accumulate on the membrane surface as gel-like aggregates, influencing the fluid-dynamic conditions and enzyme kinetic properties. "

P23, last sentence: What is meant here by "selectivity decrease"? Does this mean that a broader range of oligosaccharides passed through the membrane?
Answer: YES! In order to better clarify this point the following sentence was added page 22 line 523 "In particular a membrane selectivity decrease (a broader range of oligosaccharides passed through the membrane) of about 25 % was observed when the flux was increased from 5 to 55 L m-2h-1."

P25, Section 2.5, paragraph 1 ,L2-4: Aren't these three fields of knowledge required for any of the other processed described in this review?
Answer: YES!The sentence was deleted: ~~They are considered as emerging and very promising technologies, in which knowledge on three different fields are required: (bio)catalysis, membrane technology and reactor design.~~

P26, paragraph 1: Why is the enzyme cost more of an issue for the MBR than for the traditional enzymatic esterification process? Is more enzyme required for the former, or is it less stable in the MBR, or is it just that enzyme costs represent a higher share of total process cost because other steps (such as the separation operation shown in Fig. 6A)?
Answer: the sentence (pag 24 line 559) "the enzyme cost is considered a problem in MBR" is in general not related to the esterification process! In order to better clarify this point the sentence was modified as follows "However, the enzyme cost is considered as one of the main limitation of MBR in general, which could be reduced by the enzyme immobilization (Fjerbaek et al., 2009), because it significantly increases enzyme stability and re-use"

Minor edits:

The manuscript is riddled with misspellings, syntax errors, etc. A partial sample is listed below.
P2. Introduction, L1: Here and in several points in the manuscript, the authors misuse singular and plural terms. In this case, "is" should be "are".
Answer: corrected
P5, Section 1.1, L2: "monoxigenases".
Answer: corrected
P6, L13-14: "Thricoderma", "Clorstridium".
Answer: corrected
P6, paragraph 2, L2, Insert "bonds" ahead of "between".
Answer: corrected
P7, Section 1.2, paragraph 1, last sentence: "and/or". Can be one or the other, but not both.
Answer: corrected
P11, paragraph 2, L1-3: Rewrite sentence to active voice ("Lin and Ghazali used…").
Answer: corrected
P17, Section 2.1.3: Numerous instances of "B-glucosidase" improperly italicized.
Answer: corrected
P21, Section 2.3: "Xilanase" in section title.
Answer: corrected
P21, last line: "monosaccaride".
Answer: corrected
P22, top half of page: convert "a" and "b" in enzyme names, (e.g., "b-glucosidase") to Greek letters.
Answer: corrected
P22, L4: Separate "Larabinofuranosidase" to "L-arabinfuranosidase".
Answer: corrected
P22, paragraph 4, L2: First "were" should be "where".
Answer: corrected
P24, L1: Change "permits to overcome" to "overcomes".
Answer: corrected
P24, L8: Change "to remove" to "removal of".
Answer: corrected
P24, L13: Change "deactivate" to "deactivating".
Answer: corrected
P25, Section 2.5, paragraph 1 ,L5: Insert "with" ahead of "respect".
Answer: I think the meaning will change so I let it as it is
P25, Section 2.5, paragraph 2, L1: Correct "maily".
Answer: corrected

Highlights

1) Enzymes combined with artificial membranes in biorefinery promote process intensification
2) The use of MBRs in biorefinery permit enzymes re-use and increased stability
3) The use of MBRs promote removal of enzyme inhibitors and continuous operation
4) The use of MBR in biofuels, phytotherapics and food ingredients production was analyzed

Enzyme catalysis with artificial membranes towards process intensification in biorefinery- A review ~~Enzymes combined with membranes in biorefineries~~

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Abstract

~~The demand for sustainable alternative sources to produce biofuels, biochemicals, biomaterials, pharmaceuticals have increased worldwide.~~

~~In order to reduce the strong competition with food biomass (1st generation biorefineries), 2nd ,3rd (e.g. wood, grass, leaves, microalgae, etc.) and 4rd 4th (genetically engineered microalgae) generation biorefineries have become excellent alternatives.~~

~~This does not only mean a change in the raw material, but also in innovative production concepts based on alternative green technologies.In this scenario, sustainable downstream processes are highly desired. Among the different membrane technologies, the integration of enzymes and membranes in membrane bioreactors (MBRs) is highly interesting, since it permits process intensification, coupling bioreaction and separation. Besides, other advantages promoted by MBRs in biorefineries are thecan also promote enzymes re-use, removal of enzyme inhibitors, continuous operation with a subsequent increase in conversion and enzyme stability.~~

In this review, for the first time, the conjugation of the major types of enzymes used in biorefineries and the membrane processes to develop different configurations of MBRs, was analyzed for the production of biofuels, phytotherapics, food ingredients~~, etc~~. In particular, the aim is to critically review all the works related to the application of MBR in biorefinery, highlighting the advantages and the main drawbacks which can interfere with the development of this system at industrial scale. Alternatives strategies to overcome main limits will be also described in the different application fields, such as the use of biofunctionalized magnetic nanoparticles associated with membrane processes for enzyme re-use and membrane cleaning or the membrane fouling control by the use of integrated membrane process associated with MBR.

Keywords: membrane bioreactor, MBR, biorefinery, biocatalysis, enzymes in biorefinery

1 Introduction

Biorefineries ~~is~~are based on a wide range of technologies able to transform biomass into its simpler components (proteins, sugars, tryglycerides, etc), which can be further converted into biofuels and other chemicals

On the basis of the feedstock used~~ and the final product~~, it is possible to classify biorefineries in different generations. In the first generation, the main feedstocks are starch- or sugar-based materials: sugarcane, corn, wheat, barley, sorghum, and sunflower. ~~The high content of sugars and oil permits an easy and high production of biofuels (biodiesel, bioethanol, biogas, vegetable oil and biomethanol. However, the main problem of the first generation biorefineries is the competition with food and feed industries for land use and exploitation.~~

Although the high content of sugars permits high production of biofuels there is competition with food and feed industries for land use and exploitation(Singh et al., 2019)

Second generation biorefinery ~~concerns~~ are biofuels produced from non-food crops processing (forage, bagasse, solid waste, animal fat, wheat straw, rice straw, bagasse, cotton stalk, wheat bran, etc), and are mainly composed of lignocellulosic materials. Together with biofuel, the products could be also high added value compounds ~~(proteins, sugars, nutraceuticals etc).~~ Compared to the first generation, the second generation biorefineries is considered more eco-friendly, more cost-effective and more compatible with the societal development, since it does not exploit food resources. The third generation biorefinery concerns biofuels and biochemicals production from algal biomass (microalgae, cyanobacteria and macroalgae) (Enamala et al., 2018). The great advantages of this biomass are: independence of seasonal growth, high productivity, low CO_2 emission (Aguilar et al., 2018), no use of pesticides and herbicides in the cultivation (Ahamed & Vermette, 2008) etc. However, there are some limitations, such as high cost for cultivation and harvesting, which compromises the development at industrial scale. Life cycle analysis (LCA) studies (Cai et al., 2018) have demonstrated that in the first generation biorefineries there is a

reduction in greenhouse gas emission and fossil energy consumption, but as far as the industrial development is concerned the second generation biorefineries is more appropriate, because it is more eco-friendly, not in competition with food and cost effective. This is the reason why this review is mainly focused on second generation biorefineries.

~~In the fourth generation, biofuel and biochemicals are produced from genetically modified microalgae, with improved photosynthetic efficiency. As mentioned for the third generation, there is no competition with food, no land usage, large amount of nitrogen and carbon source, increased fermentation and hydrolysis, high yield of biofuel and biochemical. The main disadvantage is the the expensive harvesting and genetic engineering process.~~ The different steps required for the biorefinery are: harvesting, milling and crashing, transformation, separation and formulation. Membrane processes are used in many of the above mentioned steps. However, our review will focuse on transformation and separation promoted by biocatalyst and membrane separation in membrane bioreactors (MBR). MBRs in biorefineries can promote enzymes re-use, removal of enzyme inhibitors, continuous operation with a subsequent increase in conversion and enzyme stability.

~~The different steps required for the biorefinery are: harvesting, milling and crashing, transformation, separation and formulation. As illustrated in Table1, mMembrane processes are used in many of the above mentioned steps., Hhowever, our review will focuse on transformation and separation promoted by biocatalyst and membrane separation in membrane bioreactors (MBR).~~ The aim of this review is to show the potential of MBR in biorefinery, highlighting drawbacks which can limit its developmend on industrial scale, but also the innovative strategies, which seem very promising in controlling membrane fouling, enzyme re-use and stability, inhibition product removal and process integration. To reach this aim, a brief overview ~~of biomass and enzymes used in biorefinery and in conjugation with membrane processes will be given, followed by the description~~ of MBR technology will be given followed by the main applications of it in different sectors of biorefinery.

Table 1 Biorefinery steps and role of membrane processes

Genetically modified algal biomasses have improved photosynthetic efficacy, increased amount of light penetration as well as reduced photo inhibition

	1ª generation	2ª generation	3ª generation	4ªgeneration
Harvesting	✘	✘	✔	✔
Milling, crashing	✘	✘	✘ ✔	✘✔
Transformation	✔	✔	✔	✔
Separation	✔	✔	✔	✔
Formulation	✔	✔	✔	✔

✔: membrane processes can be applied

✘: no applications of membrane processes

1.1. Biomass and enzymes used in biorefineries

Biomass is the organic material derived from wood, vegetable and microbes, which is mainly composed of cellulose, hemicellulose, lignin, starch, fats, chitin, oil, etc. Lignocellulosic biomass, among all sustainable energy sources, provides a viable route to produce organic fuels. The production of liquid fuels provides besides of easy fueling and storage, low net greenhouse gas emissions and a relatively high energy density. The hydrolysis of the polysaccharides for production of liquid fuels and chemicals offers important strategic, environmental, and economic advantages. Although the cost has been historically too high compared to fossil alternatives, research over the last 20 years helped the technology to advance to the point that it is becoming economically viable.

Lignocellulosic materials are composed of lignin, cellulose and hemicellulose, with small amounts of proteins, pectins and ash (Kumar et al., 2009). This biomass includes agro-residues, forestry wastes, energy crops and wastewater of textile, wood processing and paper or pulp industries (Jönsson & Martín, 2016). For instance, the pulp and paper industries produce 500–1000 m^3 wastewater per ton of paper (Holik et al., 2006), which contains a considerable amount of cellulosic material. (Cabrera, 2017)

Cellulose is the fundamental constitutional part of vegetal material and it is organized in a systematic fibrous structure. Each fiber is constituted by repetitive units of glucose connected each other by β-1,4-glycosidic bonds forming a linear homo-polysaccharide.The smallest repetitive units of cellulose is the cellobiose, which is made by two molecules of glucose linked by a β-(1,4′) glyosidic bond. H-bond network (intramolecular and intermolecular hydrogen bond between cellulose) gives the crystalline structure of cellulose, which confers to this material it insolubility and its high resistance to enzymatic attack. The insolubility of cellulose is also conferred from its enormous chain length. The cellulose fibril is formed by ordered crystallites and low ordered non-crystalline (amorphous) domains (Chesson & Forsberg, 1997; Saini et al., 2015). Hemicellulose

connects the cellulose fibrils with lignin and it consists of highly branched repetitive units of pentoses and hexoses sugars (about 50-200 units).

Hemicelluloses are generally classified as xylans, mannans, and glucans, with xylans and mannans being the most prevalent according to the main sugar residue in the backbone. Depending on the plant species, developmental stage, and tissue type, various subclasses of hemicellulose may be found, including glucuronoxylans, arabinoxylans, linear mannans, glucomannans, galactomannans, galactoglucomannans, β-glucans, and xyloglucans.

The term "xylan" refers to all polysaccharides that have a β-(1→4)-D-xylopyranose backbone with a variety of sidechains. Xylan is the predominant hemicellulose in most plant cell walls, generally comprising about 1/3 of the total plant biomass (Prade, 1996). This compound is an amorphous polymer that is more easily hydrolyzed into its component sugars than cellulose. However, hemicellulose is typically made up of five different sugars: arabinose, galactose, glucose, mannose, and xylose as well as other components such as acetic, glucuronic, and ferulic acids (Wyman et al., 2005).

Lignin is a complex amorphous polymer composed by hydrophobic phenolic units, which surrounds the cellulose fibrils forming a complex matrix covalently attached to hemicellulose. This polymer confers high mechanical and microbial resistance to the vegetal material. In general, herbaceous plants have a very low content of lignin.

Due to the high complex structure of lignocellulosic material, the enzyme treatment is not efficient alone and it is generally preceded by a pre-treatment, in which the main aim is to reduce the complexity of lignocellulosic biomass (disruption of cellulose and lignin structure, increasing the exposure of amorphous cellulose etc.) and to facilitate the subsequent fermentation/enzymatic processes. (Kumar et al., 2009) On the basis of the different content of lignin, hemicellulose and crystalline cellulose, different pre-treatment strategies can be used (Table 2). The final aim of the pre-treatment is the production of substrate which can be converted by biocatalysis to glucose and xylose. The general strategy utilized to hydrolyze lignocellulose material into the monomer glucose

is similar to the starch hydrolysis. The only challenge in hydrolyzing cellulose is that the glucose in cellulose is linked by β-(1→4)-bonds in a crystalline structure that is far more difficult to hydrolyze than the alpha bonds in amorphous starch.

Table 2 Pre-treatments used to decrease the complexity of lignocellulosic materials.

Pre-treatment	**Mechanism**
Physical	Mechanical comminution, pyrolysis
Biological	Fungi degradation (involved enzyme are lignin peroxidases and manganese-dependent peroxidases, polyphenol oxidases, laccases, and quinosine-reducing enzymes)
Chemical	Ozonolysis, acid hydrolysis, alkaline hydrolysis
Physicochemical	Steam and fiber explosion
Electrical	Pulsed electric fields

Fig. 1 Process scheme for the valorization of ligno/cellulosic biomass.

Wood is another important source of biomass mainly divided in softwoods (plant without seeds, gymnosperms), and hardwoods (plant with seeds, angiosperms).

Starch can be mainly found in the seeds and roots, but its content is not so high in the residual biomass, since it is degraded byfrom living organisms. Besides, most of the production of starch is mainly for human nutrition.

Pectin are polysaccharides mainly composed of homogalacturonan (HG), rhamnogalacturonan (RG-I and II), and xylogalacturonan. They can be found in lignocellulosic material and are generally used as gelling agent (Khedmat et al., 2020). The oligosaccharides recovered after their hydrolysis have shown important therapeutic effects such as antioxidant, antibacterial, etc.

Lipids are another very important starting material for biofuels production due to its high content of carbon and hydrogen. They can be found in seeds and in minor quantity in vegetal material, although they are the main constituents of cell membranes. In some organisms (e.g. microalgae), they can be found as triglycerides and free fatty acids. In recent years, they have been easily

~~extracted from microalgae, when grown in stress conditions, and different articles demonstrated the possibility to fractionate/purify lipids and other bioactive compounds by membrane operations (Djamai et al., 2019; Giorno et al., 2013; Marbelia et al., 2016).~~

~~Different enzymes are involved in biomass degradation: cellulases, hemicellulases, amylases, ligninases, pectinases, lipases, proteases, monoxigenasesmonooxygenases, etc (Fig. 2).~~

~~Cellulases are groups of enzymes able to hydrolyze lignocellulosic materials and can be either complexed (as in cellulosomes) or uncomplexed and extracellular. The cellulase enzymes are a combination of three main enzymes, which act in a synergistic way: endoglucanase, exoglucanase and β-glucosidase. Cellulases can be produced by several microorganisms such as: *Trichoderma reesei, Aspergillus niger, Clostridium thermocellum (Escamilla Alvarado et al., 2017).*~~

~~They can catalyze the reaction of water with the glucose sugar molecules in lignocellulose chains to release the monomeric glucosesugar and In this hydrolysis reaction, several glucose molecules may also be released as intermediates often containing only 2 to perhaps 3 glucose sugar units. Cellulase enzymes are very specific in only catalyzing the addition of water to glucan chains, with optimum reaction conditions (pH 4.5-5 and temperature about 50°C), virtually eliminating degradation reactions. Thus, only glucose is formed via enzymatically driven hydrolysis of cellulose, with sometimes close to 100% yield. On the contrary, the hydrolysis of celluloisic material with dilute acids (e.g., 1.0% sulfuric acid) requires temperature as high as 220°C, while the acid also triggers formation of hydroxymethyl furfural as side product reducing the yield of the desired productunlike acid hydrolysis that needs high temperature and produce side products like hydroxymethyl furfural.~~

~~The cellulase enzymes are a combination of three main enzymes, which act in a synergistic way: endoglucanase, exoglucanase and β-glucosidase. Endoglucanase can hydrolyze amorphous cellulose, acting on β-1.4 linkage and producing cellooligosaccarides. Exoglucanases produces cellobiose, acting on reducing and non reducing ends, while β-glucosidase produces glucose monomer hydrolyzing cellobiose. Cellulases can be produced by several microorganisms such as:~~

~~*Thrichoderma reesei, Aspergillus niger, Clorstridium thermocellum (Escamilla - Alvarado et al., 2017).*~~

~~The monooxygenase enzymes are another class of very important enzymes, since in combination with other cellulases, they can degrade the crystalline region of cellulose (Villares et al., 2017). They can be produced by different microorganisms; however very attractiveness is the use of recombinant monooxigenases in the biofuels production (Moreau et al., 2019).~~

~~Amylases enzymes can hydrolase starch (α-amylase) and in particular the 1,4-α-D glucosidic bonds between glucose units or they can hydrolyze non-reducing ends of amylose and amylopectin (glucoamylase). In the case of starch hydrolysis, the main products are maltose, glucose and maltotriose, while for amylose and amylopectin hydrolysis just glucose can be produced.~~

~~Pectin is another important component of biomass, hydrolyzed by pectinases with the production of a galacturonic acid, well-known for its healthy properties. In particular, fruit waste, which contains pectin, is used as raw material to be treated, and therefore belongs to the second generation biomass(Ciriminna et al., 2015). An interesting review (Ciriminna et al., 2015) summarizes the worldwide extraction processes and the main companies that commercialize this product as a feedstock. Different subclasses of enzymes, such as polygalacturonase, pectin lyase, pectin methylesterase, pectate lyase belong to the pectinases class, which act in a synergic way to carry out depolymerization and de-esterification reactions.~~

~~Lipases are another important group of enzymes involved in biomass treatment (Bajaj et al., 2010) and in particular on trylglycerides hydrolysis with the production of di or monoacylglycerols, fatty acids and glycerols, but they can also carry out esterification of tryacylglicerides with the production of a mixture of alkyl esters and glycerols.~~

~~Fig. 2 Biocatalysts involved in biorefineries.~~

1.2. Integration of biocatalyst and membrane ~~process~~ operations in MBR

A membrane bioreactor ~~(MBR)~~ is a merged process, which ~~promotes separation by combining~~combines a membrane ~~process~~ operation and biocatalysis. In MBR, the membrane can have a catalytic function being the site where the ~~to support the~~ biochemical reaction ~~inside the reactor~~occurs (biocatalytic membrane reactor, BMR) or non-biocatalytic function ~~to~~ where it only ~~support~~ perform the separation process (MBR) (Giorno & Drioli, 2000; Giorno et al., 2009). In the case of BMR, the membrane itself is catalytic with the biocatalyst being immobilized within the membrane pores. (Mazzei et al., 2017b). On the basis of the membrane module location, external or internal to the reaction mixture, MBRs can be classified in side-stream or submerged configuration (Fig. ~~3~~1), respectively. In both configurations, the biocatalyst can be free or immobilized, and the strategy to supply feed and withdraw product can be either continuous and/or intermittent.

Several types of membranes and membrane processes can be combined with bioconversions (Table ~~3~~1). Membranes made of organic polymers, inorganic materials, mixed matrix components, with hydrophilic or hydrophobic character can be used (Drioli & Giorno, 2020). Symmetric or asymmetric strucures, flat-sheet, spiral-wound, tubular or capillary configuration are suitable in developing MBR. Separation based on sieving mechanism (microfiltration MF, ultrafiltration UF) also combined with Donnan exclusion (nanofiltration (NF)), or solution-diffusion (forward osmosis (FO), pervaporation (PV)), partition coefficient (membrane based solvent extraction (MBSX)), membrane emulsification (ME)), evaporation (membrane distillation (MD)) can be combined with the biocatalysis (Giorno & Drioli, 2009).

MF and UF using porous (0.1 – 10 µm) and mesoporous (2 -10 nm) membranes, respectively, are often used in combination with biocatalysis for continuous production of valuable compounds and/or treatment of streams. Continuous membrane fermentors or cell recycle membrane bioreactors are applied when the reaction involves bacteria that perform the bioconversion during the growing phase and/or large size substrates that would not be able to enter the porous matrix (Chang et al., 1994; Giorno et al., 2002). In these cases, the membrane retains the biocatalyst and the large size substrate whilst it permeates the small size products. Examples of application of these

systems include the production of carboxylic acids by fermentation of Lactobacillus bulgaricus (Choudhury & Swaminathan, 2006; Giorno et al., 2002). ~~Giorno et al. demonstrated that the mass of lactic acid produced in a cell recycle membrane bioreactor was almost doubled compared to the one produced in a batch bioreactor (Giorno et al., 2002) . This was due to the high cell density and low concentration of inhibitors tuned in the continuous system thanks to the permselective properties of the membrane.~~ In cases where the bioconversion of large size substrate macromolecules is catalyzed by enzymes in order to retain it by MF or UF, it is necessary to enlarge its size, which is often obtained by immobilizing enzymes on nanoparticles (Chang, 2018). If the substrate is small enough to enter the membrane pores, then, the biocatalyst (bacteria in vegetative stage or enzymes) can be immobilized within porous matrices and the reaction occurs within the pore void volume (Giorno & Drioli, 2000; Giorno; et al., 2017). Examples of application of this configuration in biorefinery, include production of valuable compounds ~~(such as nutraceuticals, antioxidants, anti-inflammatories)~~ and energy vectors ~~(such as bioethanol)~~ (Drioli & Giorno, 2009; Mazzei et al., 2013). The immobilization of enzyme in membranes demonstrated to increase enzyme stability (Giorno & Drioli, 2000) without necessarily affecting the enzyme catalytic activity (Mazzei et al., 2012), supposed that the microenvironment is tuned to guarantee suitable enzyme macromolecular flexibility and rigidity, water activity (Vitola et al., 2017), substrate mass transport (Giorno et al., 2006).

NF (using membranes with 0.5 – 2 nm) is usually combined with biocatalysis carried out by free enzymes and it is used to fractionate small molecular weights intermediates (Tay et al., 2018). However, some example of enzyme immobilized on NF membranes was also reported (Dizge et al., 2018). Applications include fractionation of oligosaccharides, peptides, amino acids, organic acids.

MDSX is applied to carry out bioconversions using interfacial biocatalysts (such as lipases) immobilized within the membrane where the organic/water interface is also located (Giorno et al., 2007). Field of applications include production of active ingredients ~~(such as optically pure enantiomers)~~ (Sakaki et al., 2001), processing of vegetable oils ~~(Chakraborty et al., 2012)~~.

MD and FO are mainly used for concentration of biocatalyst or molecules upstream the membrane (Goh et al., 2015; Holloway et al., 2015; Song & Liu, 2019). This is usually the case when waters coming from agro-food industries are present in diluted streams that need to be concentrated in order to reduce processing costs. PV is used in combination to bioconversions to separate alcohols from water-based mixtures (Fan et al., 2016). ME is a relatively novel membrane process able to formulate emulsions on a drop-by-drop mechanism through the membrane pores, which disperse at high throughput, a non-miscible phase into another, at low energy input. ME was proven to be a powerful technique to assist bioconversion by separating reaction product (Mazzei et al., 2010) or by formulating biocatalysts distributed at the interface (Piacentini et al., 2021).

2. Use of MBRs in biorefineries

2.1 Cellulase and membrane processeses in biorefineries

The bioprocessing of agro-food residues, such as rice and wheat straws, sugar bagasse and corn stover with 30–50% of cellulose content, are under intense research and development, with promising results and high technological readiness levels (TRL). Cellulose enzymatic hydrolysis is considered one of the most costly steps in the bioconversion of lignocellulosic biomass (Malmali et al., 2015), which involves an interfacial heterogeneity of solid cellulose substrate and cellulase enzyme adsorption~~. The mixture of cellulase enzymes appears to be more effective and with lower cost than a pure single enzyme preparation. (Bélafi-Bakó et al., 2006) There are a many studies that use cellulase from various microorganisms acting on different cellulose substrates. They~~Various studies confirmed that it is possible, via membrane technology, to retain the enzymes present in the system, while allowing the transfer of lower-molecular weight reaction products to pass through the membrane (Andrić et al., 2010a).

Table ~~4~~ 2 is a comprehensive summary of these studies, and major points are discussed in more details below. Most of the cases utilize membranes with molecular weight 10-50 kDa cut-off i~~n the range of 10-50 kDa~~ (Table 2). Usually, the reaction mixture of the substrate and enzyme is recirculated in the membrane reactor, whereas a stream with the products is withdrawn from the

permeate side. Flat sheet membranes in a side-stream configuration are prevalently used. Only in few systems, a submerged membrane hollow fiber configurations, which can be more beneficial ~~from operational point of view~~in terms of fouling control, are used.

Major challenges that limits industrial scale MBRs for cellulose hydrolysis include low substrate concentration, enzyme microbial degradation, and membrane fouling. For example, the cellulose concentration (2-5w/v%) is considered low for industrial application as it leads to low glucose concentration in the permeate (Malmali et al., 2015; Nguyenhuynh et al., 2017).

~~However, there are limitations for membrane systems in cellulose hydrolysis. For example, they operate at cellulose concentrations 2 to 5 w/v %, which are considered low for industrial scale application. This low substrate concentration leads to low glucose concentration in the permeate. In addition to these disadvantages, other potential issues are membrane fouling, and enzyme microbial degradation during recovery in liquid phase.~~

2.1.1 Discontinuous MBR and product inhibition

During cellulose hydrolysis, although a 100% yield is expected due to enzyme specificity ~~enzymatic hydrolysis of lignocelluloisic biomass is expected to provide up to 100% yield due to the enzyme cellulase specificity~~, most batch ~~wise~~ reactions could not achieve this ~~were never able to achieve such a high yield~~, due to enzyme product-inhibition. The inhibition of cellulolytic enzymes by glucose, cellobiose (Berlin et al., 2007), which are produced during saccharification (Cantarella et al., 2014; Ximenes et al., 2011), released during lignocellulosic pretreatment, is a well-known problem. This is exacerbated by ~~In addition, batch hydrolysis imparts~~ the high enzyme cost, imparted by its ~~when it is discharged~~ dischargment and ~~replaced~~replacement. The cellulase enzyme replacement contributes up to 20% of the total cost in case of bioethanol production ~~process~~ and ~50% of the entire hydrolysis step, limiting both the technological and economic feasibility of the hydrolysis process. ~~The enzyme recycling and reuse for a longer period could be beneficial for the entire process. These are the main challenges for making the hydrolysis process even more~~

~~technologically and economically feasible. (Nguyenhuynh et al., 2017) The inhibition of cellulolytic enzymes by glucose, (Berlin et al., 2007), which are produced during saccharification and phenolics (Cantarella et al., 2014; Ximenes et al., 2011), and released during lignocellulosic pretreatment, is a well-known problem.~~ A detailed analysis of the mechanisms and kinetics of the product-inhibition of cellulolytic enzymes by glucose and cellobiose has confirmed that reactors should be designed with continuous or semi-continuous product removal. As a result, numerous studies have focused on the integration of membrane bioreactors (MBRs) in biorefineries for simultaneous hydrolysis and continuous/intermittent *in-situ* product removal (Gebreyohannes et al., 2013; Mahboubi et al., 2017b; Nguyen et al., 2015).

In this section we will discuss major research findings using intermittent/discontinuous processes.

A four-fold increase in enzymatic hydrolysis of cotton cellulose with intermittent removal of the product cellobiose, by using a flat-sheet polyethersulfone membrane was achieved (Gavlighi et al., 2013). ~~In that case, the cotton-cellulose conversion after 3 days was ~19% by weight.~~ Authors achieved 19% degree of conversion after 3 days, for a reasonable feed concentration of 25 g/L.

The hydrolysis of microcrystalline pure cellulose powder was also evaluated in a tubular MBR configuration and compared with a flat-sheet MBR (Bélafi-Bakó et al., 2006). 95% of the ~~cellulose~~ cellulase was retained by membrane as estimated by dry weight measurements and only 6% of the initial enzyme activity has been observed in the permeate. Thus, the membrane sufficiently retained both the substrate and enzyme. Possibly, due to better mass transfer,~~By using microcrystalline pure cellulose powder as substrate~~, the tubular membrane gave 10% higher average conversion than the flat-sheet membrane configuration.

In another MBR (Liu et al., 2011) configuration the cellulase from *Aspergillus niger* was free in solution and retained in the MBR by a polyethersulfone ultrafiltration membrane. Also in this system a complete retention of both cellulose and cellobiase was observed.

In a recent study, a modified submerged MBR for enzymatic cellulose hydrolysis was developed (Nguyenhuynh et al., 2017). In this work the intermittent product removal was used and in the

mentioned conditions more effective UF performance with complete glucose permeation and enzyme retention up to 80% was obtained.

Qi et al. (Qi et al., 2012) examined the application of combined UF and NF for recovering the cellulase and concentrating glucose, respectively, in an integrated approach. They found that the UF membranes permitted a cellulase retention of 74%, a conversion of 84.5% and a recovery of all the glucose in the permeate. ~~The UF permeate was then concentrated (from 30.2 g/L to 110.2 g/L glucose) with NF270 membranes.~~

~~In addition to enzyme-product inhibition, the cellulose particles present in the substrate solution appear responsible for the severe fouling in such membrane bioreactors resulting in remarkable flux decline in the most of the studies (Alfani et al., 1982; Bélafi-Bakó et al., 2006; Nguyenhuynh et al., 2017). Lim and Ghazali [39] have recently studied the membrane fouling mechanism during the cellulose hydrolysis in an enzymatic reactor using the Hermia's pore blocking model. Hydrolysis has successfully converted more than 80% of the substrate into reducing sugar. The flux analysis results showed that the membrane fouling was dominated by a cake formation mechanism. The large macromolecules of the reaction mixture (substrate and enzyme) blocked the membrane pores and eventually caused the development of cake layer.~~

Although UF based MBR was effective to retain the enzyme and limit enzyme product inhibition, the system was prone to membrane fouling. As a strategy to limit membrane fouling, Lim and Ghazali (2020) used an intermittent product removal ~~strategy in order to reduce the effect of membrane fouling~~ during the continuous hydrolysis of microcystalline cellulose ~~was used~~. The removal of the product from the bioreactor using UF membrane filtration was done under two different strategies. For Strategy 1, 50% of the reaction mixture was filtered after 4 h of hydrolysis reaction to remove the reducing sugar. The recycling of the enzyme and the filtration of the hydrolysate were carried out simultaneously. The hydrolysis reaction was continued and the filtration was repeated at the 8th h~~. The filtration was re-started at the 24th h. Fresh cellulose was then added. The cycle was repeated and the filtration was performed at the 28th, 32nd, 48th, 52nd, 56th~~

~~and 72nd h.~~ For Strategy 2, the fresh substrate and citrate buffer were added at a 24 h interval, while the filtration process started at the 24th h.

Compared to the batch productivity (63% of cellulose conversion after 72 h), the intermittent product removal gave a 10x times higher productivity, due to the limited enzyme-product inhibition. The more frequent product removal, together with the enzyme recycling, was sufficient to main a reasonable reactor productivity. Table 2 also shows that most of the systems utilized side-stream MBR configuration, which enforces pumping a slurry. Recently, there is a growing effort and success in the use of submerged MBR in order to resolve this issue. A modified submerged MBR system with intermittent product removal developed recently for instance gave an effective UF performance with complete glucose permeation and up to 80% enzyme retention (Nguyenhuynh et al., 2017).

In another approach, the hydrolysis of α-cellulose was carried out in a ~~with cellulase with two different operations was carried out with in batch and~~ submerged continuous MBR. Since an ~~microfiltration~~ MF membrane was used in the submerged system, a pre-holding time was allowed in order to promote a better binding between enzyme and substrate (Malmali et al., 2015). The continuous hydrolysis with in-situ product removal gave an order of magnitude higher rate of glucose production relative to batch process, due to enzyme product-inhibition. ~~In a batch catalysis of carboxymethyl cellulose was observed that using enzyme cellulase immobilized on magnetic nanoparticles, the enzyme efficiency, i.e. the ratio of product mass over enzyme mass, was limited to about 15 mg/mg$_{enz}$(Gebreyohannes et al., 2018).~~ On the other hand, the biocatalysis of carboxymethyl cellulose in an MBR ~~membrane bioreactor~~ equipped with ~~microfiltration~~ MF and enzyme immobilized on magnetic nanoparticles led to a constant reaction rate over time, and 50% higher enzyme efficiency, due to in-situ product removal (Gebreyohannes et al., 2018). The use of biofunctionalized nanoparticles have the inherent issue of nanoparticle aggregation at high concentration. Hence, designing the system under reaction rate limited regime can prevent mass transfer resistance due to particle aggregation and the subsequent loss of biocatalytic

efficiency.~~which helped to avoid the enzyme product inhibition.~~ In addition to *in-situ* product removal, the use of~~A~~ a cocktail of synergistically performing different cellulytic enzymes can be an effective strategy to reduce the extent of ~~in order to prevent~~ the enzyme-product inhibition ~~in both batch and continuous hydrolysis was used~~(Gebreyohannes et al., 2018; Lozano et al., 2014). ~~When batch hydrolysis was run with endoglucanase only, the monomer to oligomer ratio decreased over time due to inhibition of the enzyme by cellobiose. On the contrary, w~~When the hydrolysis of carboxymethyl cellulose was run with a mixture of endoglucanase and β-glucosidase, ~~the monomer-oligomer ratio significantly increased over time, especially with higher β-glucosidase content. Nevertheless, this batch hydrolysis still suffers from β-glucosidase inhibition by glucose. However, the use of a similar enzyme cocktail~~ in an MBR configuration ~~helped to simultaneously increase the~~ higher monomer to oligomer ratio, was obtained due to absence of ~~while also preventing the~~ cellobiohydrolase and β-glucosidase inhibition by cellobiose and the ~~β-glucosidase inhibition by~~and glucose, respectively (Gebreyohannes et al., 2018). Not only the use of mixture of these enzymes but also~~Similarly, the use of~~ an appropriate ratio of cellulase and cellobiase is highly imperative to achieve~~(38 and128 U/ g cellulose) during the hydrolysis of regenerated cellulose, led also to a~~ rapid cellobiose hydrolysis and prevented the cellulase inhibition (Lozano et al., 2014).

2.1.2 Continuously fed MBR, limitation to low MWCO membrane and operational conditions

As shown in Table 2, most MBRs for cellulose hydrolysis are operated with a separated bioreactor and pumping of the slurry across the membrane for ultimate retention/recycling of the unreacted substrate and enzyme, while allowing permeation of glucose. In order to retain the 60 kDa cellulase enzyme (Suurnäkki et al., 2000), the membrane molecular weight cut-off used in this application is often limited to about 10 kDa (Giorno & Drioli, 2000; Tian et al., 2015). Andrić et al. (2010b) have previously indicated that an appropriate MBR design for continuous enzymatic hydrolysis with *in-situ* product removal is crucial. However, a side-stream configuration is a limiting factor to successful large scale applications, since pumping a slurry imparts a significant operating cost

(Roche et al., 2009; Stickel et al., 2009). Moreover, low MWCO membranes require high transmembrane pressure and leads to significant membrane fouling (Lim & Ghazali, 2020; Lozano et al., 2014; Mahboubi et al., 2017a). While a continuously fed MBR could face severe membrane fouling, owing to the enzyme retention and simultaneous product removal, a continuously/intermittently fed system can have better productivity.

For instance, a corn stover pre-treated by soaking in 15 wt% aqueous ammonia incubated with a cellulase loading of 60 FPU per initial cellulose was used to compare the performance difference among batch, continuously fed and intermittently fed MBR. Intermittent addition of 5 g/L cellulose every 8 h, gave a total glucose of 1.94 and 1.88 times higher than batch reactor without MBR and continuously fed MBR, respectively.

~~For instance, the aqueous amino acid pre-treated corn stover, with a cellulase loading of 60 FPU per initial cellulose and by intermittent addition of 5 g/L cellulose every 8 h, gave 1.88 times higher a total glucose of 1.94 and 1.88 times higher than batch reactor without MBR and continuously fed MBR., respectively. In addition, to increase reactor productivity, tThe intermittent feeding strategy also was able to increased the product concentration from 0.5 g/L to about 2 g/L.~~ ~~Nevertheless~~Yet, the obtained product concentration in many of the studies is considerably low (0.2-20 g/L,~~ see Table 52~~) (Gebreyohannes et al., 2018; Lim & Ghazali, 2020; Lozano et al., 2014; Zhang et al., 2011).~~ for~~ Since the desired concentration for subsequent fermentation to ethanol, falls between 150 to 250 g/L glucose (Malmali et al., 2015), a significant energy is consumed in pre-concentration. Increasing the substrate concentration specially when using high MWCO membrane can be one strategy to achieve a higher product concentration (Malmali et al., 2015).~~which often requires 150 to 250 g/L glucose (Malmali et al., 2015). As expected, increasing the substrate concentration an increase of the product was obtained (Table 5), although the contribution of the enzyme amount was not considered, since in the studied articles pure enzymes or mixture of several enzymes and different enzyme units were used.~~

In all these discussions, it was difficult to elucidate the contribution of the enzyme, as the type, amount and units of the enzymes used were different. ~~since in the studied articles, pure enzymes or mixture of several enzymes and different enzyme units were used.~~

~~The frequency of intermittent product removal and substrate feeding are also important factors, as they both can dictate the rate of membrane fouling. A more frequent product withdrawal was beneficial to avoid the enzyme product inhibition.~~ ~~Up to 51% flux decline due to fouling was observed during the UF of hydrolyzed wheat straw, though this never hampered passage of reducing sugars.~~ Various strategies have been employed to alleviate the issue of membrane fouling. A good example could be application of electro-ultrafiltration (EUF) ~~was employed under different operating conditions,~~ during the filtration of pre-hydrolyzed acid pre-treated wheat straw to mitigate the membrane fouling. EUF is a method, where a differential electric field is applied across the membrane to achieve electrostatic repulsion of membrane foulants (Hakimhashemi et al., 2012). ~~The results showed that EUF was effective to reduce concentration polarization and enhance the filtration flux in recycling cellulase.~~ The flux when the system was fed with 2% w/v lignocellulosic hydrolyzate increased by a factor of 4.4 ~~at 836 V/m~~ at room temperature, compared to that without electric field ~~This work shows that, under appropriate operating conditions, EUF can efficiently recycle cellulase from lignocellulosic hydrolyzate and thus substantially reduce the hydrolysis cost.~~ (Chen et al., 2013)**.** ~~Intermittent feeding and product withdrawal have already been discussed as a strategy to increase MBR productivity. However, controlling the frequency of intermittent product removal and substrate feeding are also important factors, since they dictate the rate of membrane fouling.~~

Moreover, intensification of the hydrolysis step with the subsequent~~Combined processes, in which saccarification followed by~~ fermentation process in a simultaneous saccharification and fermentation (SSF) ~~is carried out,~~ seems to be the most promising strategy to increase overall productivity. ~~systems since they permit process intensification.~~ . The potential application of such hybridized system was recently shown by~~An example of the potentiality of the system~~ (Mahboubi

et al., 2020) ~~was recently published~~, ~~in which a double staged immersed MBR promoted continuous, stable and long-term (264 h) saccarification-filtration system and co-fermentation filtration of straw slurry~~.

The cellulose hydrolysis using MBR often requires low solid loading or low solid loading rate and continuous dilution in order to reduce the extent of membrane fouling, the enzyme product-inhibition and the difficulty of pumping a concentrated slurry. In order to resolve the issue of pumping slurry, a submerged MBR with a 10 kDa UF membrane was designed. Although the UF membrane was successful in retaining the enzyme (97%) and avoided pumping slurry, the cost for the pressurized reactor is considerable, while the membrane fouling was still severe (Zhang et al., 2011).

Alternatively, a submerged MBR integrating an MF membrane was employed (Malmali et al., 2015), which avoids pumping cellulose slurry. ~~The membrane was able to reject the cellulose particles and enzymes adsorbed onto the cellulose.~~ Owing to the use of MF, a high initial cellulose loading (100 and 150 g/L) was used, which are significantly higher than the cellulose loading observed in most MBRs (see Table 2). Higher substrate loading ensured higher glucose concentration; hence, the steady-state glucose concentration was 10-15 g/L. These values are significantly higher than the concentration obtained in the various UF systems. One of this systems' disadvantages is enzyme loss through the membrane. However, the extent of enzyme loss was limited by the introduction of pre-holding time that provided sufficient time for the enzyme to attach onto the cellulose. As a result, compared to the very high initial enzyme loading (50 mg/g cellulose), the rate of enzyme addition during continuous operation was either 4 or 10 mg enzyme/g cellulose injected. In addition, the use of higher cellulose loading ensured more enzyme retention. ~~MBRs with a pre-holding time revealed two distinctive zones: a rapid drop in glucose concentration during pre-holding time followed by quasi steady-state values during the continuous glucose withdraw, owing to absence of product inhibition in the latter step. The glucose productivity in MF is also significantly higher than UF, due to the higher imparted flux. Since controlling a continuous~~

system is more complicated than batch, to maximize the glucose production in this system, optimization of enzyme and substrate loading, pre-holding time, holding time (ratio of reactor volume to permeate flow rate), rate of mixing are highly imperative.

The cellulytic hydrolysis using MBR often requires low solid loading or low solid loading rate and continuous dilution in order to reduce the extent of membrane fouling, the enzyme product-inhibition and the difficulty of pumping a concentrated slurry. In order to resolve the issue of pumping slurry, a submerged MBR with a 10 kDa UF membrane was designed. Although the UF membrane was successful in retaining the enzyme (97%) and avoided pumping slurry, the cost for the pressurized reactor is considerable, while the membrane fouling was still severe. (Zhang et al., 2011)

Alternatively, a submerged MBR integrating a microfiltration membrane was employed (Malmali et al., 2015). The submerged MF membrane avoided pumping cellulose slurry. The membrane was able to reject the cellulose particles and enzymes attached to them. Owing to the use of MF, a high initial cellulose loading (100 and 150 g/L) was used, which are significantly higher than the cellulose loading observed in most MBRs (see Table 4). Higher substrate loading ensured higher glucose concentration, hence the steady-state glucose concentration (10-15 g/L). These values are significantly higher than the concentration obtained in the UF system. One of these system disadvantages was the enzyme losses through the pore of the membrane. This was improved by the introduction of pre-holding time that provided sufficient time for the enzyme to attach to the cellulose particles. As a result, compared to the very high initial enzyme loading (50 mg/g cellulose), the rate of enzyme addition during continuous operation was either 4 or 10 mg enzyme/g cellulose injected. Also the use of higher cellulose loading ensured more enzyme retention. MBRs with a pre-holding time revealed two distinctive zones: a rapid drop in glucose concentration during pre-holding time followed by quasi steady-state values during the continuous glucose withdraw, owing to absence of product inhibition. The glucose productivity in MF is also significantly higher than UF, due to the higher imparted flux. Since controlling a continuous system is more

~~complicated than batch, to maximize the glucose production in this system, optimization of enzyme and substrate loading, pre-holding time, holding time (ratio of reactor volume to permeate flow rate), rate of mixing are highly imperative. Since MF can retain cellulose bound to cellulase particles only, it is less interesting to employ it in a side-stream configuration. (Malmali et al., 2015)~~

2.1.3 Biocatalytic membrane reactors in ~~cellulase~~ cellulose hydrolysis

Commercial cellulase enzyme is often a cocktail of cellulolytic enzymes that include endo/exo glucanase, cellobiohydrolase and β-glucosidase. However this mixture generally exhibits low β-glucosidase activity (Rosgaard et al., 2006). Therefore, the hydrolysis by endo-glucanase mainly favors the production of oligomers such as cellobiose and cellotriose. As a result, Gebreyohannes, Dharmjeet (Gebreyohannes et al., 2018) for instance obtained 50-60% higher oligomer productivity than monomers when using an MF membrane system with immobilized enzyme. Over production of cellobiose on the one hand causes enzyme product inhibition, while on the other hand it may cause loss of significant amount of it to the permeate. In order to limit this problem, it is imperative to supplement the system with additional β-glucosidase (Andrić et al., 2010b). ~~This will eventually help with hydrolyzing cellobiose to glucose, which avoids severe enzyme product inhibition by cellobiose and also limits the amount of cellobiose leaching into the permeate.~~ Especially co-immobilization of these enzymes in a biocatalytic membrane reactor (BMR) configuration is highly beneficial. Accordingly, both Gebreyohannes et al. (2018) and Song et al. (2016a) observed a significantly improved monomer productivity by co-immobilization of cellulase and β-glucosidase in a BMR (4 times higher) and STR respectively. Enzyme immobilization is also a good strategy to shift from UF membrane based MBRs to MF based BMRs that will eventually ensure a higher volumetric reactor productivity.~~Accordingly, both Gebreyohannes et al. (Gebreyohannes et al., 2018) and (Song et al., 2016a) observed a significantly improved monomer~~

~~productivity by co-immobilization of cellulase and β-glucosidase in a BMR (4 times higher) and STR respectively.~~

For instance, the natural tendency of enzyme to be adsorbed by cellulose, often a concern for enzyme efficiency loss, was taken as an advantage in order to retain the enzyme in 0.6 µ MF equipped submerged MBR for cellulose hydrolysis.While this system requires significant pre-holding time in order to ensure sufficient adsorption, the loss of enzyme is still unavoidable

~~The attachment of enzyme to the cellulose particles was shown as one strategy to employ MF in a submerged MBR for cellulose hydrolysis; however the loss of enzyme is still unavoidable.~~ In this case, membranes with immobilized enzyme in BMR configuration can be beneficial. ~~As a result, apart from a few studies (Ishihara et al., 1991; Knutsen & Davis, 2004), there is a lack of data on the performance of highly porous membrane reactors for enzymatic conversion of lignocellulose.~~ Although the issue of enzyme leakage can be resolved through confining the enzyme on to the membrane or carrier particle, BMRs are less often used (Andrić et al., 2010a). ~~To date, only few industrial applications of immobilized enzymes in general exist. (Di Cosimo et al., 2013)~~ However, since enzyme immobilization can contribute to the development of sustainable processes, it has substantial potential to be used in industrial lignocellulose-to-ethanol conversion. (Chang et al., 2011; Rodrigues et al., 2017)

~~BMRs with the cellulase entrapped in the membrane matrix (Chang et al., 2011), adsorbed to the membrane (Bayramoğlu et al., 2010; Bélafi-Bakó et al., 2006) or covalently bound to the membrane (Mazzei et al., 2009; Wu et al., 2005) have long been studied. Enzymes hydrolyse substrate to facilitate permeation through the membrane. In the longer period, the loss of enzyme activity through deactivation or wash-out will likely occur while the inevitable membrane fouling even if the enzyme is still active will nonetheless demand for membrane cleaning. However, none of the traditional enzyme immobilization strategies can allow membrane cleaning or replacing damaged immobilized enzyme.~~

600 ~~In this regard, a~~ A very recent strategy of biocatalytic systems is to immobilize enzymes on
601 superparamagnetic nanoparticles (NP^{SP}). These particles afterwards are reversibly immobilized on a
602 microporous membrane using an external magnetic field in a system named superparamagnetic
603 biocatalytic membrane reactor (BMR^{SP}) (Gebreyohannes et al., 2015; Gebreyohannes et al., 2017).
604 The immobilization of the enzyme on the NP^{SP} can improved stability, activity along with easy
605 recovery using an external magnetic force (Ladole et al., 2017; Lupoi & Smith, 2011; Song et al.,
606 2016b; Xu et al., 2011). Due to the possibility of using MF membrane with immobilized enzyme, it
607 was possible to achieve constant glucose productivity at high solid loading (2-10 wt% CMC), high
608 solid loading rate (3-6 g/h ~~up to 15-30 L/m^2 h~~) and negligible rate of fouling (0.008 bar/min) in a
609 submerged system. This is an immense improvement of the lignocelluloisic hydrolysis, which is
610 generally limited to UF membranes to retain the enzymes ~~with the disadvantages of severe fouling,~~
611 ~~leading to high transmembrane pressure and often low solid loading and solid loading rate~~
612 (Gebreyohannes et al., 2018).
613 On the basis of the reported studies on enzymatic ~~about the use of cellulose for~~ cellulose hydrolysis,
614 enzyme stability, enzyme turnover, membrane fouling and product concentration still remain open
615 challenges. The reactor design must be fully considered, particularly to limit the enzyme cost,
616 which contributes 25-30% operational cost (Guo et al., 2018). Side-stream ~~The main~~ MBR
617 configuration, which ~~used is the one that~~ combines free enzyme carrying out the hydrolysis in bulk
618 and a membrane that removes the reaction products, is by far the most investigated. In ~~the~~ this
619 ~~mentioned~~ configuration, the enzyme compartimentalization promoted by membrane process,
620 guarantees enzyme re-use and product inhibiton limitation, showing huge potential in operational
621 cost reduction. Since MF can only retain enzymes compartmentalized to membrane or carrier
622 particles, it is less interesting to employ it in a side-stream configuration (Malmali et al., 2015).
623 Over all, use of membrane was effective in retaining the enzyme and preventing enzyme-product
624 inhibition through intermittent/continuous product removal. Though dictated by the frequency of
625 feeding and product withdrawal, this strategy also helps to mitigate membrane fouling. In terms

626 configuration, a hybridization of hydrolysis with fermentation could be a way forward towards
627 industrialization. While a submerged MF equipped MBR with immobilized enzyme could be an
628 optimal strategy to increase MBRs volumetric productivity.~~important potentiality in the reduction~~
629 ~~of the operational cost~~.
630
631 **2.2. β-glucosidase and membrane process in biorefinery**
632 ~~As reported in section 1.1 (Biomass and enzyme used in biorefineries),~~ β-glucosidase is a key
633 enzyme in determining efficiency of cellulase for biomass hydrolysis, but recently it has also gained
634 attention for its ability to hydrolyze glycosidic substrates from vegetal biomass to produce
635 aglyconic compounds, which have important therapeutic properties (Mazzei et al., 2012; Mazzei et
636 al., 2009; Ranieri et al., 2018). The use of membrane bioreactors in the production of aglyconic
637 compounds solved several problems: the continuous removal of the inhibiton product (glucose)
638 from the reaction site, the extraction of the water unstable aglycones in organic solvents by
639 multhiphasic MBR, (Mazzei et al., 2010) and the enzyme reuse. On the basis of the problem
640 treated (e.g. glucose inhibition, aglycones extraction, kinetic study etc), β-glucosidase was
641 entrapped on polymeric membranes (Mazzei et al., 2012; Mazzei et al., 2009) or covalently
642 attached on ceramic membrane (Fig ~~4A~~2A) (Mazzei et al., 2012)(Fig 2B) (Ranieri et al., 2018). By
643 using both biocatalytic polymeric and ceramic membranes, it was possible to produce an intensified
644 system, in which the production/extraction of the aglycone in a pure organic solvent was promoted
645 (Fig. 2). In the mentioned system, the aglycone extraction process is obtained by recirculating a
646 pure organic solvent, in which the compound is soluble, in the lumen of a tubular membrane. When
647 the aqueous phase, coming from the biocatalytic membrane and containing the product, it reaches
648 the membrane lumen, on the basis of the membrane emulsification process an unstable emulsion is
649 produced, which permits the aglycone extraction from the aqueous to the organic phase (Mazzei et
650 al., 2010)(Fig. 2 a and b). Due to membrane processes modularity, the intensified MBR/ME system
651 with an MF/UF process (Conidi et al., 2014) or with two steps of membrane emulsification

652 (Piacentini et al., 2019) was easily integrated (Fig.3). In the first work, olive mill waste water
653 (OMWW) pre-treated by MF/UF steps and containing the glycosidic substrate (oleuropein) was fed
654 to the intensified process, obtaining the same degree of conversion ~~of~~ when pure substrate was used
655 (Fig. 3A). In the second system, in addition to the production/extraction of oleuropein aglycone, its
656 encapsulation in hydrophilic polymeric (Fig. 3B) or hydrophobic solid lipid particles (Fig. 3C) was
657 also promoted (Piacentini et al., 2019).
658 Recently, a further improvement of the system in terms of conversion (93%) by using the enzyme
659 free in solution and promoting aglycone extraction by ME process (Fig. 3D) was obtained (Mazzei
660 et al., 2020). The role of the membrane, in this system, was to retain the enzyme and to wash out
661 the glucose from the reaction mixture. This permitted to re-use the biocatalyst for five consecutive
662 reaction cycles, with no decay in conversion. In the two last mentioned systems, olive leaves as
663 source of biomass to obtain the glycosidic substrate were used.
664
665 **2.3. ~~Xilanase~~ Xylanase and MBR in biorefineries**
666 Xylan is the second most abundant renewable compound on earth and a sustainable technology
667 which permits the recovery/fractionation of xylo-oligosaccharides (XOS) and monosaccharide from
668 xylan is one of the current priorities in the research related to biorefineries. On the basis of the type
669 and content of substituents within the xylan structure, the synergistic action of xylanase (in
670 particular endo-1,4-β-xylanase and β-xylosidase) and other debranching enzyme (α-L-
671 arabinofuranosidases, α-glucuronosidase, acetyl xylan esterases and ferulic acid esterases) is
672 generally needed. However, due to the product inhibition on the xylanases enzymes a separation
673 step to isolate the biocatalyst is necessary, particularly if a ~~productive~~ large scale and a continuous
674 process is needed.
675 A lot of recent articles propose membrane bioreactor technology to overcome the limits given by
676 product inhibition (Andrić et al., 2010a; Nabarlatz et al., 2007; Pinelo et al., 2009; Sueb et al.,
677 2017) and to simultaneously purify the product from the reaction mixture.

However, it must be considered that the substrate tends to accumulate on the membrane surface as gel-like aggregates, influencing the fluid-dynamic conditions and enzyme kinetic properties.

In the work carried out by Sueb et al. (2017) the effect of fouling due to particle deposition was evaluated by different configuration of MBRs. The MBRs configuration used were: a) reaction (endo-1,4-b-xylanase and β-xylosidase, free state) and filtration (1 kDa PES membrane) in the same system; b) xylanase (free state) reaction and filtration in a MBR and a further enzymatic reaction of the permeate by xylosidase in a STR; c) both enzymes present in a stirred tank reactor and a subsequent filtration process. Reaction with both enzymes followed by UF (configuration C) was the optimal configuration, which permitted at least 40% higher xylan hydrolysis than the cascade configuration.

In the work carried out by Acosta-Fernández et al. (2020), a membrane with higher nominal molecular weight cut-off (10 kDa) was used starting from xylan from coffee parchment. In the mentioned research the enzyme free in solution or immobilized on magnetic nanoparticles, in 2 STRs and in 2 MBRs, were compared. Results demonstrated that by using the MBRs configurations a continuous production of xylooligosaccharides, with the molecular weight distribution in the range of prebiotic sugars (X1−X20) was obtained. By optimizing the fluid-dynamic conditions a high conversion can be also achieved at high substrate concentration. Besides, the unchanged apparent Km demonstrated that the enzyme immobilization procedure did not alter the affinity of the enzyme for the substrate and it was even improved when membrane process was present, since it promoted a continuous removal of inhibition products from the reaction mixture.

Biofunctionalized magnetic nanoparticles were also coupled to an organic-inorganic hybrid membrane (were magnetic nanoparticles were used as nanofillers) to develop a nano-inspired, magnetic-responsive enzyme membrane (micro) reactor (Gebreyohannes & Giorno, 2015). In this system xylanase and pectinase as model biocatalysts were used to control membrane fouling. The system permitted 75% reduction in membrane filtration resistance through the membrane surface cleaning~~, thanks to the action of biofunctionalized nanoparticle present on the membrane surface~~.

An integrated membrane process was also proposed by González-Muñoz et al. (2008), in which liquors containing xylan-derived products from rice husk was firstly treated with diafiltration ~~(1 kDa ceramic membrane)~~ and then by MBR to obtain and purify low molecular weight arabino-xylooligosaccharides (AXOS). Also in this study the various MBR configurations were studied. ~~In the first reactor, the reaction and products separation simultaneously occurred, while in the other the reaction was carried out in a STR and it was followed by a membrane process.~~ The best configuration in terms of productivity (93.3% recovery yield vs 75.8%) was the one in which the catalysis was carried out simultaneously with the separation process.

2.4. Pectinase and MBR in biorefineries

Pectin is a complex polymer of carbohydrates present in the cell wall of the main higher plants. In recent years, pectic biomass is considered as an important source of feedstock, because it contains a low lignin concentration and in some industrial process (e.g. juice filtration) is considered a waste material, which can be valorized through hydrolysis process.

It can be also used as starting source to produce galacturonic acid, which is a~~s~~ raw material in food, pharmaceutical and cosmetic industry, due to its important ~~pharmaceutical and cosmetic~~ properties or for pectin-derived oligosaccharides (POS). POS are an emerging class of prebiotic, but they can also have important therapeutic properties such as: ability to induce apoptosis in human colon cancer cells, anti-inflammatory and antiobesity properties, etc (Gómez et al., 2016). On the basis of the different pectic biomass used, oligosaccharides with different structure can be obtained such as arabinogalacto-oligosaccharides, arabinoxylooligosaccharides, galacto-oligosaccharides etc. Pectin hydrolysis can be carried out by both chemical and enzymatic methods, but as frequently observed the enzymatic methodology offers several advantages such as reaction in mild conditions avoiding corrosion, selective hydrolysis and higher reaction yield. However the pectic enzymes generally suffer from product inhibition of the monomer (galaturonic acid). For this reason, a separation process after hydrolysis is highly desired. This is the reason why membrane processes are generally

coupled with enzymatic hydrolysis for pectin in MBR systems, which permit the continuous POS production, enzyme re-use and conversion increase due to inhibition product removal(Gómez et al., 2016). MBR technology for pectin hydrolysis is currently used by both immobilized and non-immobilized enzyme, although the most used configuration is with free enzyme recirculated in the retantate side (Table 3) (Alkorta et al., 1995; Bélafi-Bakó et al., 2007; Rodriguez-Nogales et al., 2008; Rodríguez-Nogales et al., 2005). In the last mentioned systems, both flat-sheet and hollow fiber membranes made of different materials were used. Two kind of reactors are used: sequential batch reactor and filtration (discontinuous) or simultaneous batch filtration process (continuous). In the first case, the reaction occurs in a first step after a certain incubation time without product separation. The membrane process is used in a second step to carry out the purification. To avoid the excessive production of monosaccharides, small amount of biocatalyst is used for this reason and the enzyme concentration to achieve the highest conversion is one of the most studied parameters(Mountzouris et al., 2002; Torras et al., 2008). The incubation time is another parameter frequently studied to control the MW of the products, but the non-specific enzyme cleavage does not permit to control it. As a result, batch reactors coupled with membrane processes are not suitable for further application for the production of POS, since the final product have a wide MW distribution (Moure et al., 2006). Strategies for final products separation are based on the use of different membrane separation steps to obtain the different fractions of the product. Córdova et al. (2017) used three different steps of nanofiltration for oligosaccharides purification after hydrolysis in order to obtain products of target properties grouped in the desired MW range.

Nevertheless, important viscosity reduction of pectin solution in the MBR with free enzyme also without further purification by membrane processes is achieved, which is very useful in systems in which a viscous solution must be treated (e.g. filtration of fruit juice or olive mill waste water) and pectin causes membrane fouling (Gebreyohannes et al., 2013).

~~In the continuous MBR in which free enzyme is used, the reaction and separation occurs simultaneously; the enzyme is retained together with larger substrate molecules while small product~~

~~are continuously removed. In these systems, the retention time is the most important parameter that controls the final size and distribution of the product(Su et al., 2020) (Su et al., 2020).~~ In the work carried out by Baldassarre et al. (2018), a discontinuous (used as pre-treatment) and a continuous membrane reactor with free enzyme were used. This permitted to increase the volumetric productivity up to five times, demonstrating a real advantage respect to the traditional batch reactor. In the continuous MBR the process was intensified, but the flow through the membrane was lower than discontinuous systems, since large molecules tend to deposit on the membrane surface enhancing transmembrane resistance. Nabarlatz et al. (2007) demonstrated that a high solute flux during oligosaccarides fractionation caused an increase of concentration polarization and an increased retention of low MW compounds. In particular a membrane selectivity decrease (a broader range of oligosaccharides passed through the membrane) of about 25 % was observed when the flux was increased from 5 to 55 L $m^{-2}h^{-1}$.

Enzyme immobilization on membranes for POS production ~~permits to~~ overcomes a lot of problems related to both enzyme re-use and stability, targeted production of tailored products, fast POS removal and hence limiting monomer production. Nevertheless, few studies are currently applied for pectin hydrolysis in which BMRs are used. This can be due to additional problems due to enzyme immobilization (steric hindrance, enzyme aggregation) and/or enzyme deactivation due to chemical cleaning and disinfection of the biocatalytic membrane. Gebreyohannes et al. (2016) demonstrated that immobilizing the pectinase on magnetic nanoparticles, subsequently dispersed on the membrane surface by a magnetic field, permitted ~~to remove~~removal of the enzyme when necessary (e.g. membrane washing) and to distribute it avoiding steric hindrance ~~and~~ improving enzyme kinetic performance. The use of biofunctionalized particles coupled with membrane process is ~~increasing very much~~widely employed now (Donato et al., 2012; Vitola et al., 2017; Vitola et al., 2019), since it permits to recover the catalyst at the end of the process, the possibility to clean the membrane with solvent without ~~deactivate~~ deactivating the enzyme and to keep

781 unaltered the chemical-physical and morphological structure of the membrane, generally modified
782 during chemical biofunctionalization.
783
784 **2.5. Lipase and MBR in biorefineries**
785 Membrane processes and in particular MBR are innovative systems for biodiesel production and
786 can be used both in esterification, transesterification and biodiesel refining. ~~They are considered as~~
787 ~~emerging and very promising technologies, in which knowledge on three different fields are~~
788 ~~required: (bio)catalysis, membrane technology and reactor design. Although their advantages with~~
789 ~~respect to the traditional esterification systems (batch reactors, and plug flow reactors) are well~~
790 ~~known, some drawbacks (e.g. enzyme cost, stability, yield, membrane fouling) must be better~~
791 ~~studied in order to fully compete with traditional systemthem at industrial scale (Table 4).~~
792 The involvement of lipase in biorefineries is mainly in transesterification of tryalcylgricerides to
793 produce fatty acid (m)ethyl esters (FA(M)EE). The enzymatic esterification process generally
794 involves the presence of the lipase (free or immobilized) extracted from different microorganisms
795 (*Pseudomonas fluorescens, Rhizopus Oryzae, Candida rugosa and Pseudomonas cepacia etc.*), an
796 alcohol (ethanol or methanol) and a source of triglycerides, which could be vegetable oils, non-
797 edible oils (e.g. Jatropha), waste cooking oil or animal greases, microalgal oil etc (Badenes et al.,
798 2013). Compared to the chemical process, biological esterification is highly advantageous, since it
799 promotes high conversion in mild operative conditions. Besides, in the enzymatic
800 transesterification, no soaps are produced, which imply the absence of further washing steps, with
801 the reduction of production costs and wastewater. The innovation of MBR in the enzymatic
802 esterification processes is also due to the process intensification (reaction and separation in a single
803 unit) ~~(Fig.4)~~ which also significantly reduce the production steps and the system compactness with
804 respect to the traditional methods.
805

However, the enzyme cost is considered as one of the main limitation of MBR in general, which could be reduced by the enzyme ~~recycle or~~ immobilization (Fjerbaek et al., 2009), because it significantly increases enzyme stability and re-use. This is in fact the trend observed in recent literature related to MBR and transesterification process (Table 5); where the enzyme is almost always immobilized within polymeric membranes (~~by~~ mainly by covalent attachment).

Another important problem to overcome in MBR is the enzyme deactivation due to the interaction with methanol or ethanol. In particular, a molar ratio of methanol/oil higher than 1/2 causes irreversible enzyme denaturation (Su et al., 2015)(Su et al., 2020). Besides, the glycerol produced during the transesterification process, being more soluble in water, limits the interaction of the enzyme with the substrate, forming a film around the enzyme. This film does not permit the interaction with the hydrophobic substrate, with a consecutive conversion decrease. To overcome this process, different strategies were proposed, such as continuous addition of methanol, several methods for methanol supply (in oil or in water), selective removal or glycerol etc. (Belafi-Bako et al., 2002). Within the different strategies, the use of two-phase separated membrane reactors, widely applied in MBR with lipase, seems one of the most promising (Aghababaie et al., 2019). In the work carried out by Ko et al. (2012a), a two-phase MBR permitted a stepwise addition of methanol and a selective removal of glycerol, thanks to a regenerated UF membrane, coupled with a stirred tank reactor (STR). In this case, the membrane role was to supply and remove methanol and glycerol respectively, but it also worked as a contactor between the hydrophilic and hydrophobic phase (Fig.5a). In the two-phase MBR developed by Aghababaie et al. (2019) (Fig.5b) an additional role of the membrane is to retain the biocatalyst, which is in the oil phase. In both systems it was possible to reach a high conversion degree and stability.

3. Challenges and future perspective on the use of MBR in biorefinery

The main drawbacks which hindered the development of MBR in biorefinery industries are mainly the low enzyme stability and the membrane fouling. To address these issues, strategies also

proposed in this review, must be taken into account, mainly related to the selection of membrane material, operative conditions optimization and reactor engineering design. In particular:

- the conjugation of biofunctionalized magnetic nanoparticle with membrane processes can introduce an innovative strategy to selectively remove the biocatalyst when fouling occurs. This will permit cyclic membrane cleaning with solvents or backflushig, which are generally damaging for the enzyme.
- The use of estremophiles enzyme, which can tolerate high temperature could alleviate cake-layer formation on the membrane, increasing the stability of the biocatalytic membrane.
- The introduction of integrated membrane processes associated with MBR or cascade enzymatic reactions in separated MBRs could be also interesting strategies to pre-treat the stream before the enzymatic reaction, permitting membrane foulig and enzyme reaction to be checked in separated steps.
- Another interesting approach is the possible use of microfiltration membranes with immobilized enzyme in a submerged configuration, which can ensure large volumetric productivity.

In order to fully apply the mentioned strategies in future applications, the integration between membrane science, genetic engineering, and chemical engineering is needed.

4. Conclusions

~~There is an urgent need to exploit alternative routes to reveal the true potential of waste materials and to produce goods of higher quality from this waste. Efficient and sustainable technologies and production processes in biorefineries should become part of this strategy.~~

~~Membrane processes, and in particular MBRs, are generally recognized as efficient, selective, precise, flexible and intensified technologies, that integrate conversion and separation processes in the same system.~~

~~In this review, the efficiency~~ The use of MBRs in biorefineries for the first time was critically analyzed. ~~The cases of c~~Carbohydrate hydrolysis, ~~(e.g. cellulose, hemicellulose etc),~~ biodiesel production ~~(lipase)~~, aglycones ~~phytotherapics~~ production ~~(beta-glucosidase)~~, POS and galacturonic acid production ~~(pectinase)~~ and XOS production were described and critically reviewed.

~~The biocatalytic systems covered here indicate that~~ In all the analysed sectors MBRs ~~form a very promising technology, since it~~ promote~~s~~ continuous reaction system, enzyme re-use and removal of inhibiting products, while increasing the system efficiency. ~~In order t~~To promote the development of MBRs on a larger scale some drawbacks ~~(low enzyme stability and membrane fouling)~~ of this technology must be considered. Innovative strategies proposed in this review ~~(e.g. use of biofunctionalized nanoparticles, use of integrated membrane processes etc.)~~, can promote advances in membrane saving, membrane fouling control and enzyme stability improvement.

~~MBRs are in total alignment with green chemistry principles and they can easily be adopted in biorefineries, since the reactant and product mass transfer can be controlled, enhancing yields and conversions, as well as minimizing solvent use and maximizing the biomass exploitation.~~

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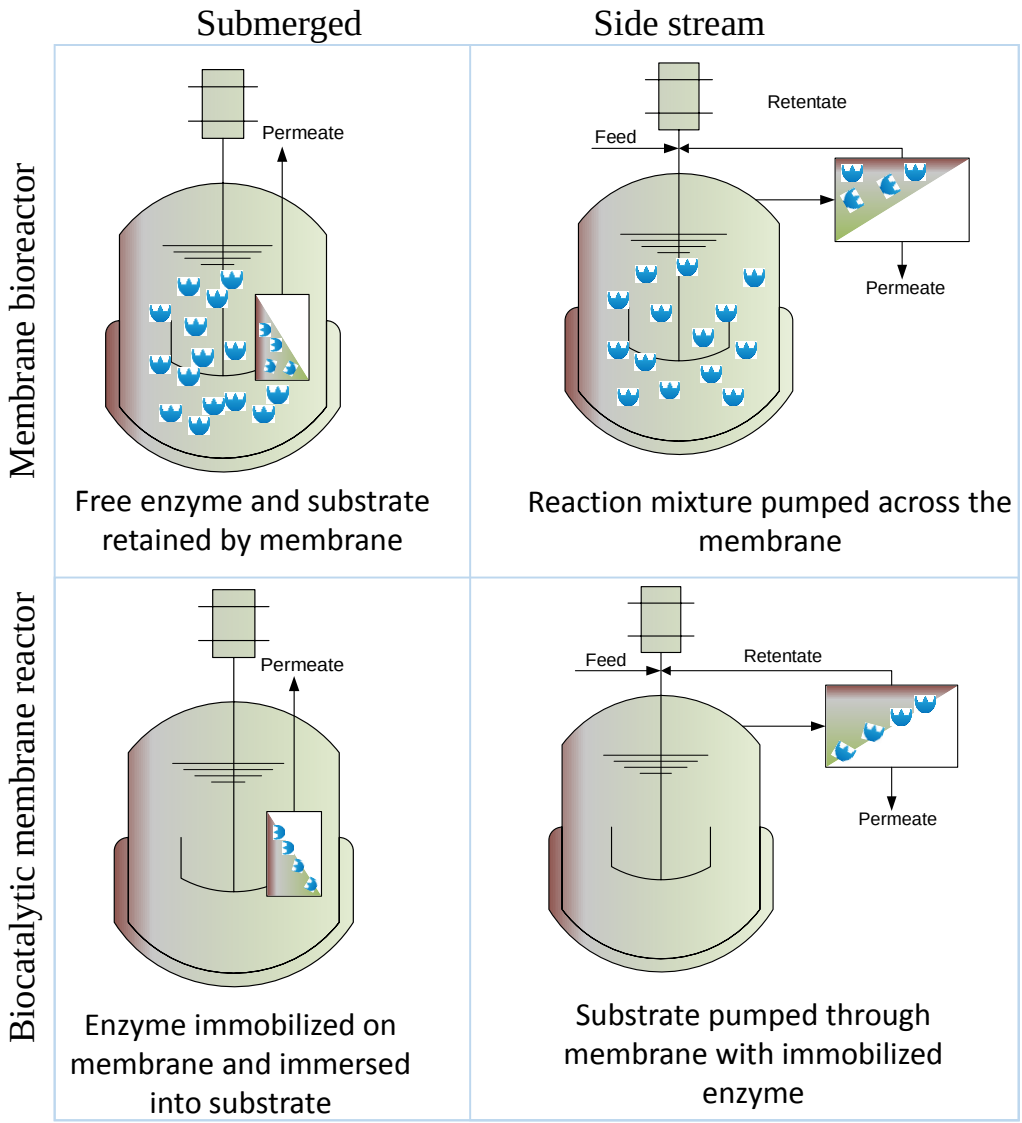


Fig. 1 Schematic representation of membrane bioreactor (MBR) and biocatalytic membrane reactor (BMR) in side-stream and submerged configuration. In the MBR the enzyme is free, while in the BMR the enzyme is immobilized.

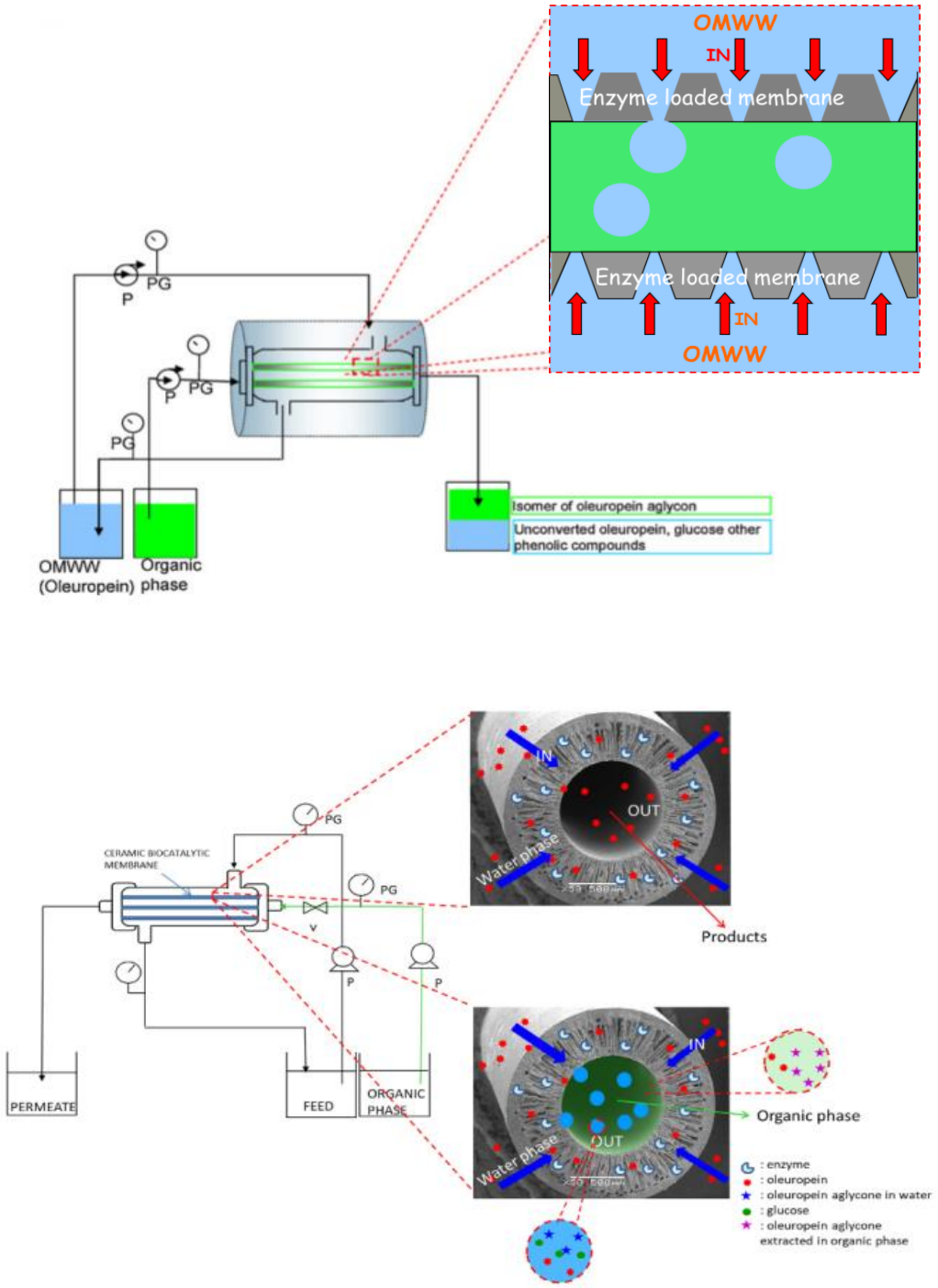
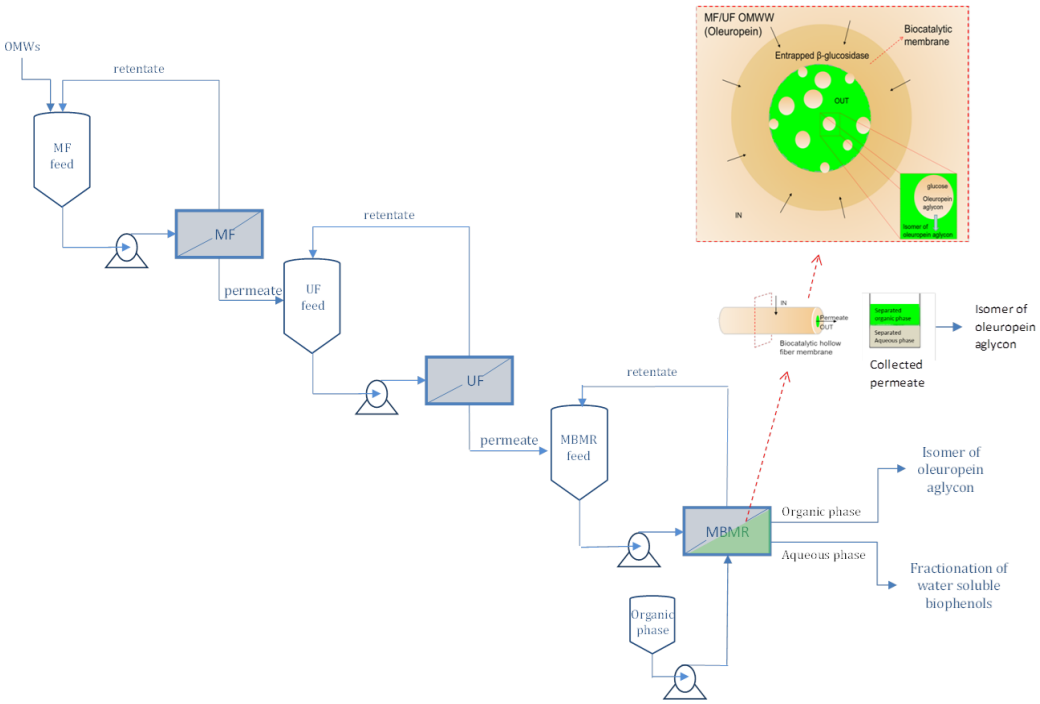
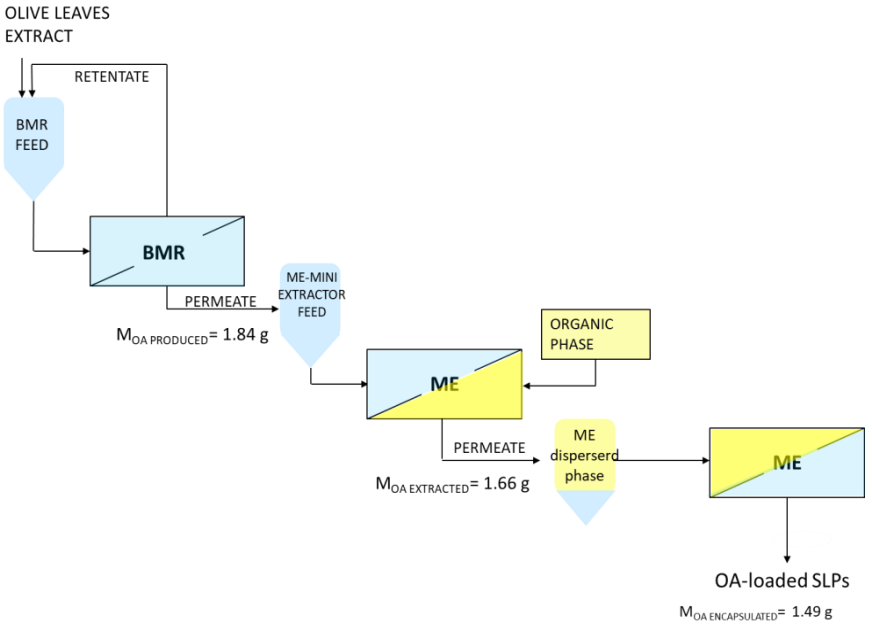


Fig. 2 Intensified membrane processes, in which MBR and membrane emulsification were coupled in a multhipashic system to promote production/extraction (in organic solvent) of aglycone. A: use of commercial polymeric membrane and physical enzyme immobilization, adapted from (Mazzei et al., 2012) with the permission of Copyright (2021) Elsevier; B: use of home-made ceramic membranes and covalent enzyme immobilization reprinted with permission from (Ranieri et al., 2018) with the permission of Copyright (2021). OA: oleuropein aglycone (product of oleuropein hydrolysis by β-glucosidase action), OMWW: olive mill waste water

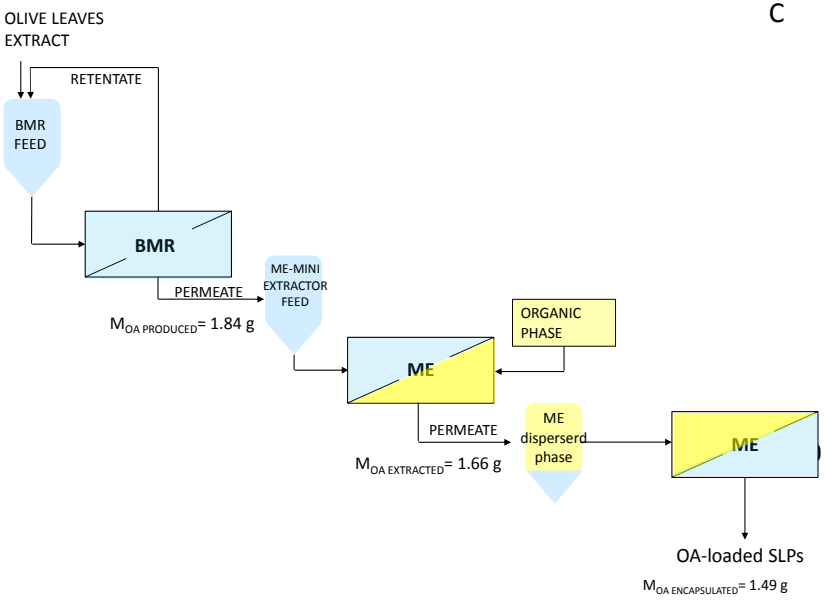
A


OMWs
retentate
MF feed
MF
permeate
UF feed
retentate
UF
permeate
MBMR feed
retentate
MBMR
Organic phase
Aqueous phase
Isomer of oleuropein aglycon
Fractionation of water soluble biophenols
Organic phase
MF/UF OMWW (Oleuropein)
Entrapped β-glucosidase
Biocatalytic membrane
OUT
IN
Biocatalytic hollow fiber membrane
Collected permeate
Isomer of oleuropein aglycon

B


OLIVE LEAVES EXTRACT
RETENTATE
BMR FEED
BMR
PERMEATE
$M_{OA\ PRODUCED}$= 1.84 g
ME-MINI EXTRACTOR FEED
ORGANIC PHASE
ME
PERMEATE
$M_{OA\ EXTRACTED}$= 1.66 g
ME dispersed phase
ME
OA-loaded SLPs
$M_{OA\ ENCAPSULATED}$= 1.49 g

C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ME dispersed phase
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OA-loaded SLPs
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D

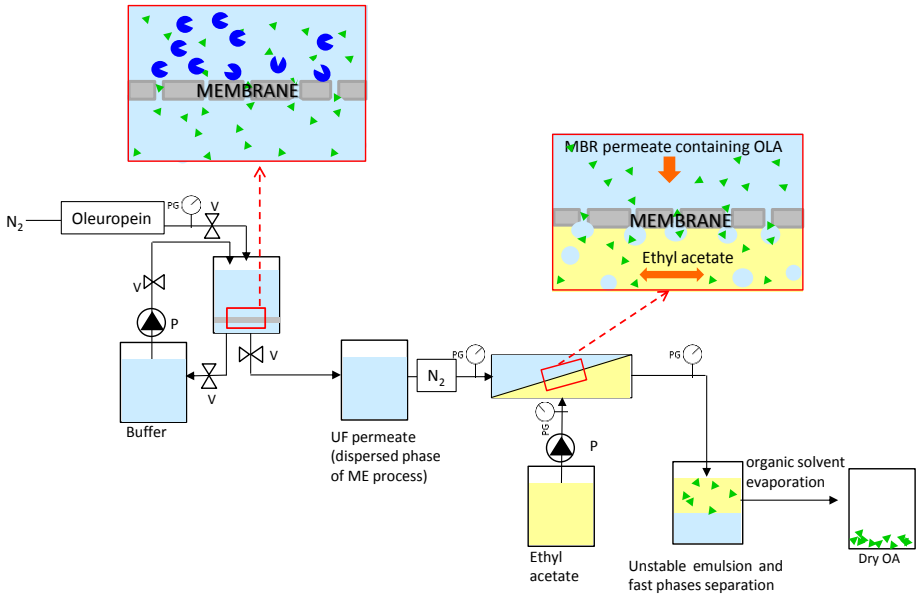
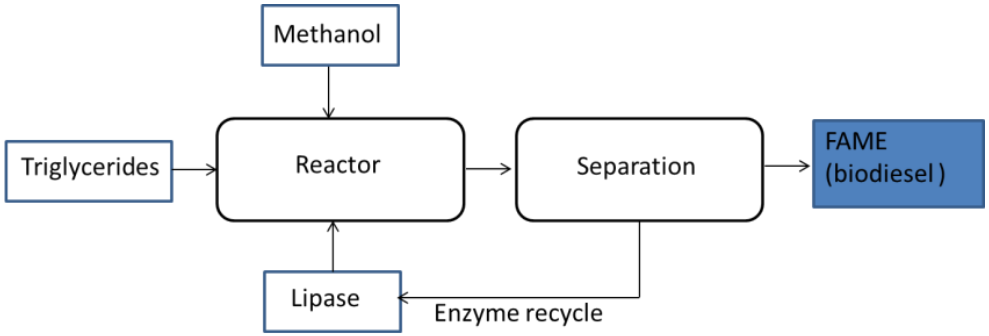
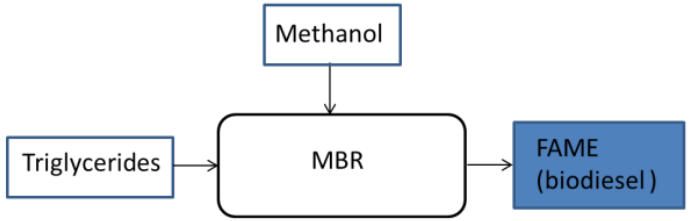

MEMBRANE
MBR permeate containing OLA
MEMBRANE
Ethyl acetate
N_2
Oleuropein
PG
V
P
Buffer
UF permeate (dispersed phase of ME process)
N_2
PG
P
Ethyl acetate
PG
organic solvent evaporation
Dry OA
Unstable emulsion and fast phases separation

Fig. 3 Multhiphasic membrane bioreactor integrated with different membrane processes for the production of aglycone or formulated aglycone starting from different biomass. A) MBR integration with MF and UF process starting from olive mill waste (OMW). Reprinted with permission from (Conidi et al., 2014). Copyright (2021) Elsevier; B) MBR integration with two steps of membrane emulsification processes to produce solid lipid particles (SLP) containing oleuropein aglycone, starting from olive leaves. Reprinted with permission from (Piacentini et al., 2019). Copyright (2021) American Chemical Society; C) MBR integration with membrane emulsification processes to produce PVA particles containing oleuropein aglycone starting from olive leaves; Reprinted with permission from (Piacentini et al., 2019). Copyright (2021) American Chemical Society D) Integration of MBR with membrane emulsification process to produce aglycone from olive leaves reprinted from (Mazzei et al 2020)(CC-BY 4.0 licence). OA: oleuropein aglycone (product of oleuropein hydrolysis by β-glucosidase action)

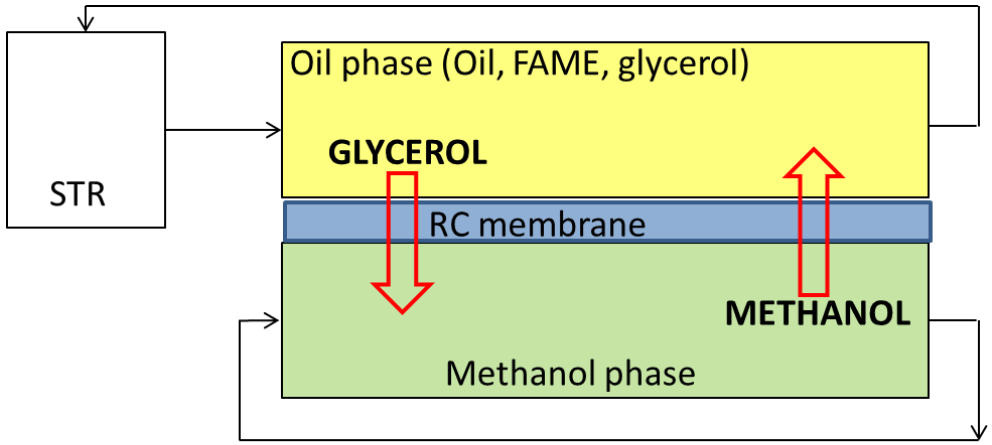


A

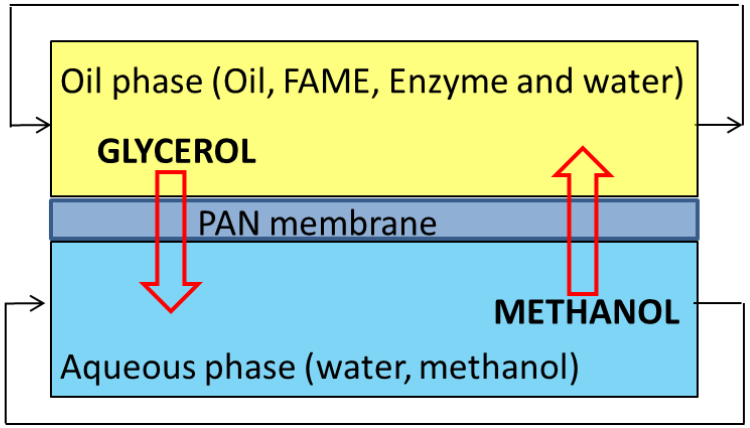


B

Fig. 4 Different steps involved in biodiesel production with traditional enzymatic esterification processes (A) and with MBR (B).



A



B

Fig. 5 Scheme of glycerol removal and methanol supply to two-phase MBR. A) system developed by Ko et al. (Ko et al., 2012b); B) system developed by Aghababaie et al. (Aghababaie et al., 2019).

Table 1 Membranes and membrane reactors in combination with enzymes in biorefinery.

Type of membrane	Membrane process	Role of membrane	Biocatalyst form	Type of Reactor	Ref.
Porous, hydrophilic	~~Microfiltration (~~MF~~)~~	Retain /recycle biocatalyst (microorganism, enzyme). Clarify stream	Free bacteria	Cell-recycle Membrane Bio-Reactor (MBR)	~~(Chang et al., 1994;~~ Giorno et al., 2002) (Choudhury & Swaminathan, 2006~~;~~ ~~Giorno et al., 2002~~)
			Enzyme immobilized on particles	Enzyme-loaded-particles recycle MBR	(Chang, 2018)
			Enzyme immobilized on membrane	Enzyme-loaded Biocatalytic Membrane Reactor (BMR)	(Giorno & Drioli, 2000; Giorno & Drioli, 2009; Giorno; et al., 2017; Mazzei et al., 2017a; Mazzei et al., 2013)
Mesoporous, hydrophilic	~~Ultrafiltration (~~UF~~)~~	Retain / recycle biocatalyst (enzyme). Remove inhibitors, products	Free enzyme	Enzyme-recycle MBR	(~~Giorno & Drioli, 2000~~; ~~Giorno; et al., 2017~~) (Drioli & Giorno, 2009; Mazzei et al., 2013) (Giorno et al., 2006) (Giorno; et al., 2017; Vitola et al., 2017)
			Immobilized enzyme	Enzyme-loaded BMR	(Giorno & Drioli, 2000; Giorno; et al., 2017) (Drioli & Giorno, 2009; Mazzei et al., 2013) (Giorno et al., 2006) (Vitola et al., 2017)

Microporous, hydrophilic	~~Nanofiltration (~~NF~~)~~	Fractionate, separate small molecular weight molecules	Free enzyme, immobilized enzyme	Enzyme-recycle MBR	(Chon et al., 2012)
			Immobilized enzyme	Enzyme-loaded BMR	(Dizge et al., 2018)
Porous, mesoporous, hydrophilic, hydrophobic	~~Membrane-Based Solvent Extraction (~~MBSX~~)~~	Assist/implement interfacial reactions in biphasic systems. Extract molecules	Immobilized enzyme	Enzyme-loaded BMR	(Giorno et al., 2007; Sakaki et al., 2001)
Porous, hydrophobic	~~Membrane Distillation (~~MD~~)~~	Concentrate molecules	Free bacteria	Cell-recycle MBR	(Goh et al., 2015)
			Free enzyme	Enzyme-recycle MBR	
Dense, hydrophilic	~~Forward Osmosis (~~FO~~)~~	Concentrate molecules	Free bacteria	Cell-recycle MBR	(Holloway et al., 2015; Song & Liu, 2019)
			Free enzyme	Enzyme-recycle MBR	
Dense, Hydrophilic	~~Pervaporation (~~PV~~)~~	Separate product, remove water	Free bacteria	Cell-recycle MBR	(Fan et al., 2016)
			Free enzyme	Enzyme-recycle MBR	
			Free enzyme	Enzyme-recycle MBR	
Porous, hydrophilic, hydrophobic	~~Membrane Emulsification (~~ME)	Enzyme distribution at O/W or W/O interface on droplets/particles surface	Immobilized enzyme	Enzyme-loaded-particles recycle MBR	(Mazzei et al., 2010; Piacentini et al., 2021)
		Solvent extraction via high throughput droplets formation		Enzyme-loaded BMR	

MF: microfiltration; NF: nanoflitration; MBSX: membrane based solvent extraction; MD: membrane distillation; FFO: forward osmosis; PV: pervaporation; ME:membrane emulsification

Table 2. Enzymatic hydrolysis of cellulose in MBRs.

Enzyme source	Enzyme content	Membrane				Feed	Conversion (%)	Feed concentration	Product concentration	Ref.
		Commercial name	Material[a]	Type[b]	MWCO (kDa)					
Trichoderma viride		Amicon PM 30	PES	FS	30		76	30%	n.d.	(Ghose & Kostick, 1970)
Trichoderma viride		Amicon PM 10	PES	FS	10		70	15 g/L	n.d.	(Howell & Stuck, 1975)
Trichoderma viride		Amicon XM50, Romicon XM50	PAN/ PVC PAN	FS HF	50		91	n.d	n.d.	(Henley et al., 1980)
Trichoderma viride	0.033 mg/mL	Amicon PM 10	PES	FS	10	Microcryst. cellulose	n.d.	1.1 g/L	2-90 mg/L	(Alfani et al., 1982)
Trichoderma reesei, Aspergillus niger		BM100	PA	FS	n.d.		50-80	n.d.	25.7 g/g	(Ohlson et al., 1984)
Trichoderma reesei	n.d.	Fitevig 500N NADIR type polymeric		HF FS	n.d. 30	Microcryst. cellulose powder	48-53	2.5% (w/v)	3.7-6.5 g /h dm^3	(Bélafi-Bakó et al., 2006)
Trichoderma reesei	n.d.	n.d.	PES	FS	10	Oil palm empty fruit bunch	n.d.	20 g/L	2-4 g/L	(Ghazali et al., 2017)
Aspergillus niger	1.5 g/L	n.d.	PES	FS	10	Sodium carboxy methyl cellulose	40-90	1.5 g/L	1.2 g/L	(Liu et al., 2011)
Trichoderma reesei	n.d	n.d.	PES	FS	10	Microcryst. cellulose	80	5-20 g/L	4.4-12.2 g/L	(Lim & Ghazali, 2020a)
Trichoderma	1.36 g/L	n.d.	PES	FS	10	Microcrystalline	80	10 g/L	5.48-	(Lim &

reesei						cellulose			6.45 g/L	Ghazali, 2020b)
Cellulase Cellic Ctec2	n.d.	~~n.d.~~	PES	FS	0.3 µm	Dilute-acid pretreated wheat straw	70-80%	14.0 ± 1.5 g/L	14.65 ± 0.59 g/L	(Mahboubi et al., 2020)
n.d.	3% w/w enzyme to substrate ratio	~~membrane type 146 (Satorius Stedim Biotech GmbH)~~	PES	FS	10	Microcryst. cellulose	n.d.	10% w/v	7.6 g/L	(Nguyenhuynh et al., 2017)
n.d.	0.7 g/l of α-amylase and 0.42 g/l of amyloglucosidase	~~n.d.~~	Commercial polydimethylsiloxane/polyethyleneterephthalate/polyimide (PDMS/PET/PI)	FS	n.d.	Broomcorn seed flour	n.d.	45 g/l	25.5 g/L	(Farahi et al., 2018)
n.d.	0.5 g/L	~~NPO30 membrane (Microdyn Nadir)~~	PES	FS	10	*α*-cellulose	45	10 g/L	2-8 g/L	(Abels et al., 2013)
Trichoderma reesei	4 g/L	~~Carbosep M5~~	ZrO_2	FS	10	Olive mill solid residue	45	n.d.	2-11 g/L	(Mameri et al., 2000)
n.d.	20 FPU/g cellulose	~~PES5 PES10 PES30~~	n.d.	FS	5 10 30	Steam exploded wheat straw	84.5	10% w/v	26.5-30.4 g/L	(Qi et al., 2012)
Trichoderma reesei	20 to 80mg/g substrate	~~PES 5 (Sepro)~~	PES	FS	5	Waste paper	67.4	20-100 g/L	12-50 g/L	(Rad et al., 2017)
Trichoderma reesei	20 FPU/g substrate	~~n.d.~~	PS	HF	10	Steam-exploded rice straw	n.d.	125-185 g/L	15-35 g/L	(Yang et al., 2006)
Trichoderma reesei	20 FPU/g substrate	~~n.d.~~	PS	HF	10	Steam-exploded corn stalk	85 (%)	100 g/L	10-30 g/L	(Yang et al., 2009)
Trichoderma longibrachiatum	*20 FPU/g dry mass*		-cation exchange membrane	FS	-DF20 - 10	acid treated wheat straw	50.3 (%)	0.5-10%	n.d.	(Chen et al., 2013)

			-PES							
Crude cellulase powder			PS	HF	30	CO_2 laser treated corn Stover	-			(Chen et al., 2013)[c]
Trichoderma reesei ATCC 26,921 (Crosslinked aggregates of Cellulase)		~~Whatman® NucleporeTM~~	PC	-	/0.22 µm	carboxy methyl cellulose (CMC)	54 (%)	20 g/L	0.5-2 g/L	(Nguyen et al., 2015)
Novozyme cellulase enzyme *(Safizym cl®)*	317.24 mg proteins/mL	~~Laval ETNA membranes~~	-	-	10, 20	carboxy methyl cellulose (CMC)	1.4 mM glucose	2.5 g/L	n.d.	(Cantarella et 2014)
Trichoderma reesei (cellulase Spezyme CP and β-Glucosidase (Novozyme 188)			PS		10	Pretreatd corn stover	-82% (batch) -94% (continuous)	15 g/L	10-30 g/L	(Zhang et al., 2011)
Cellulase			PVA	Electrospun PVA/cellulase nanofibers	-	Carboxymethyl cellulose		2% w/v	n.d.	(Wu et al., 2005)
Trichoderma reesei		-	PVDF	FS	0.2 µm	Carboxymethyl cellulose	0.9 mM	0.5 wt%	90-160 mg/L	(Gebreyohannes et al., 2018)
Cellic CTec2			PES	FS	0.62 µm	α-cellulose	0.08-0.11 mM	100-150 g/L	40-100 g/L	(Malmali et al., 2015)
Cellulase from Trichoderma reesei and cellobiase from *A. niger*			PES	TUBULAR	10	microcrystalline cellulose	113 mM	0.8 -2 w/v %	19.8 g/L	(Lozano et al., 2014)

[a] PES: polyethersulfone; PAN: polyacrylonitrile; PA: polyamide; PS: polysulfone; PC: polycarbonate.
[b] FS, flat-sheet; HF, hollow fiber
n.d., no data available in most cases, pH 4.8-5.0 and temperature 40-50^{o}C

Table 3 Use of MBR in pectin hydrolysis.

Pectin source	Enzyme	Enzyme status	Product/work aim	Membrane cut-off (kDa)/pore size (μm)	Membrane material	Reference
~~Citrus~~	~~Pectic lyase~~	~~F~~	~~POS/~~	~~10/~~	~~PS~~	~~(Alkorta et al., 1995)~~
~~Apple~~	~~Endo-polygalacturonase~~	~~F~~	~~POS/~~	~~10/~~	~~not reported~~	~~(Olano-Martin et al., 2001)~~
Apple pomace	Endopectidase, polygalacturonase	F	fouling control	10	PS	(Rodriguez-Nogales et al., 2008)
Sugar beet, black currant, red currant	Polygalacturonase from *Aspergillus niger*	F	galacturonic acid/	45/	PES	(Kiss et al., 2009)
Commercial pectin	Polygalacturonase from *Aspergillus niger*	F	galacturonic acid/ study of enzyme inhibition	30/	RC	(Bélafi-Bakó et al., 2007)
Onion skin	Viscozyme (mixture of enzymes)	F	POS/	10/	PS	(Baldassarre et al., 2018)
Lemon peels	Pectinex Ultra SP-L, pectinases from *Aspergillus aculeatus* and Pectinase 62 L	F	POS/	1/	RC	(Gómez et al., 2016)
Sugar beet	Viscozyme L,	F	POS/	10/	PS	(Elst et al., 2018)
Citrus pectin	Polygalacturonase from A.niger	IMM	POS/	/0.05−0.1	titania	(Szaniawski & Spencer, 1996)
Olive mill waste water	pectinex 3XL	IMM	/pectin hydrolysis	/0.4	PE	(Gebreyohannes et al., 2013)
Citrus fruit pectin	polygalacturonase	IMM	/membrane fouling	/0.1	PVDF	(Gebreyohannes et al., 2016)

PS: polysulphone, PES: polyethersulphone, RC: regenerated cellulose, PE: polyethylene, PVDF: polyvinylidene fluoride, IMM: immobilized, F: free

Table 4 Advantages of MBR compared to traditional biofuels production and MBR aspects that must be improved.

Advantages of MBR compared to traditional biofuel production	**Need for improvement**
Continuous operation	Biocatalyst stability
Generation of high quality biodiesel	*Ad hoc* designed membrane for different applications
Intensify the contact between reactants and catalyst	Control of membrane fouling
Can compartmentalize unreacted triglycerides	Membrane stability
Selective removal of the product during transesterification reaction	
Control the addition of reactants to the reaction mixtures	
Biocatalyst re-use	
Avoid enzyme blocking by inhibition products	
Process integration/intensification (catalysis and separation in the same system)	
Easy integration with other processes	
Easy scale-up	
Eco-friendly technology, since can carry out transesterification process in mild conditions	

Table 4 MBR systems for bioediesel production.

Enzyme	Enzyme status /Immobilization	Membrane	Membrane (kDa)/pore size (µm)	TAG source	Alcohol	Conversion (%)	Stability (days)	Ref.
Lipase from *Candida sp. 99–125*	IMM/ adsorption	textile	-	salad oil and waste oil	MeOH in n-hexane	96	more than 20	(Nie et al., 2006)
Lipase from *Candida sp.* 99–125	IMM/ covalent	textile	-	lard	MeOH	85	7.5	(Lu et al., 2007)
Lipase from *P. fluorescens*	IMM/ adsorption	PES	300/	triolein	MeOH	80	12	(Machsun et al., 2010)
Lipase from *P. fluorescens*	IMM/ covalent	PVDF	/0.45	soybean oil	MeOH in n-hexane	95	7	(Kuo et al., 2013)
Lipase from *P. cepacea*	IMM/ covalent	PAN	-	soybean oil	MeOH	90	10	(Li et al., 2019)
Lipase B form *C. antarctica* l (CalB)	IMM/ covalent	RC	10, 25, 50/	soybean oil	MeOH	97.5	-	(Ko et al., 2012b)
Lipase from *C. rugosa* (Amano AY-30)	IMM/ covalent	PVDF	/0.45	soybean oil	MeOH	97 and 95,	7	(Kuo et al., 2013)
Lipase from *Mucor miehei*	IMM/ covalent	PES	/0.65	sunflower seeds oil	Bu-OH	100	missing data	(Handayani et al., 2016)
Lipase from *C.rugosa*	F/-	PAN	100/	*Eruca sativa* oil.	MeOH	100	3	Aghababaie et al., 2019)
Lipase B from *C. antarctica*	IMM/ covalent	PAN	-	soybean oil	MeOH	80	12.5	(Li et al., 2019)
Lipase from *T. lanuginosus*	F/-	PAN	/0.2	Sunflower oil	MeOH	-	-	Sokač et al. 2020
Lipase	IMM	PES	/0.001	Karanja oil	EtOH	88	-	Kumar 2021

PES: polyethersulphone: PVDF: polyvinylidene fluoride, PAN: polyacrylonitrile, RC: regenerated cellulose, IMM: immobilized, F: free, MeOH: methanol

Enzyme catalysis with artificial membranes towards process intensification in biorefinery- A review

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Abstract

In this review, for the first time, the conjugation of the major types of enzymes used in biorefineries and the membrane processes to develop different configurations of MBRs, was analyzed for the production of biofuels, phytotherapics, food ingredients~~, etc~~. In particular, the aim is to critically review all the works related to the application of MBR in biorefinery, highlighting the advantages and the main drawbacks which can interfere with the development of this system at industrial scale. Alternatives strategies to overcome main limits will be also described in the different application fields, such as the use of biofunctionalized magnetic nanoparticles associated with membrane processes for enzyme re-use and membrane cleaning or the membrane fouling control by the use of integrated membrane process associated with MBR.

Keywords: membrane bioreactor, MBR, biorefinery, biocatalysis, enzymes in biorefinery

1 Introduction

Biorefineries are based on a wide range of technologies able to transform biomass into its simpler components (proteins, sugars, tryglycerides, etc), which can be further converted into biofuels and other chemicals. On the basis of the feedstock use, it is possible to classify biorefineries in different generations. In the first generation, the main feedstocks are starch- or sugar-based materials: sugarcane, corn, wheat, barley, sorghum, and sunflower.

Although the high content of sugars permits high production of biofuels there is competition with food and feed industries for land use and exploitation (Singh et al.,

2019). Second generation biorefinery are biofuels produced from non-food crops processing (forage, bagasse, solid waste, animal fat, wheat straw, rice straw, bagasse, cotton stalk, wheat bran, etc), and are mainly composed of lignocellulosic materials. Together with biofuel, the products could be also high added value compounds. Compared to the first generation, the second generation biorefineries is considered more eco-friendly, more cost-effective and more compatible with the societal development, since it does not exploit food resources. The third generation biorefinery concerns biofuels and biochemicals production from algal biomass (microalgae, cyanobacteria and macroalgae)(Enamala et al., 2018). The great advantages of this biomass are: independence of seasonal growth, high productivity, low CO_2 emission (Aguilar et al., 2018), no use of pesticides and herbicides in the cultivation (Ahamed & Vermette, 2008) etc. However, there are some limitations, such as high cost for cultivation and harvesting, which compromises the development at industrial scale. Life cycle analysis (LCA) studies (Cai et al., 2018) have demonstrated that in the first generation biorefineries there is a reduction in greenhouse gas emission and fossil energy consumption, but as far as the industrial development is concerned the second generation biorefineries is more appropriate, because it is more eco-friendly, not in competition with food and cost effective. This is the reason why this review is mainly focused on second generation biorefineries.

The different steps required for the biorefinery are: harvesting, milling and crashing, transformation, separation and formulation. Membrane processes are used in many of the above mentioned steps. However, our review will focuse on transformation and separation promoted by biocatalyst and membrane separation in membrane bioreactors (MBR). MBRs in biorefineries can promote enzymes re-use, removal of enzyme

inhibitors, continuous operation with a subsequent increase in conversion and enzyme stability. The aim of this review is to show the potential of MBR in biorefinery, highlighting drawbacks which can limit its developmend on industrial scale, but also the innovative strategies, which seem very promising in controlling membrane fouling, enzyme re-use and stability, inhibition product removal and process integration. To reach this aim, a brief overview of MBR technology will be given, followed by the main applications of it in different sectors of biorefinery.

1.2. Integration of biocatalyst and membrane operations in MBR

A membrane bioreactor is a merged process, which combines a membrane operation and biocatalysis. In MBR, the membrane can have a catalytic function being the site where the biochemical reaction occurs (biocatalytic membrane reactor, BMR) or non-biocatalytic function where it only perform the separation process (MBR) (Giorno & Drioli, 2000; Giorno et al., 2009). In the case of BMR, the membrane itself is catalytic with the biocatalyst being immobilized within the membrane pores. (Mazzei et al., 2017b). On the basis of the membrane module location, external or internal to the reaction mixture, MBRs can be classified in side-stream or submerged configuration (Fig. 1), respectively. In both configurations, the biocatalyst can be free or immobilized, and the strategy to supply feed and withdraw product can be either continuous and/or intermittent. Several types of membranes and membrane processes can be combined with bioconversions (Table 1). Membranes made of organic polymers, inorganic materials, mixed matrix components, with hydrophilic or hydrophobic character can be used (Drioli & Giorno, 2020). Symmetric or asymmetric strucures, flat-sheet, spiral-wound, tubular or capillary configuration are suitable in developing MBR. Separation

based on sieving mechanism (microfiltration MF, ultrafiltration UF) also combined with Donnan exclusion (nanofiltration (NF)), or solution-diffusion (forward osmosis (FO), pervaporation (PV)), partition coefficient (membrane based solvent extraction (MBSX)), membrane emulsification (ME)), evaporation (membrane distillation (MD)) can be combined with the biocatalysis (Giorno & Drioli, 2009).

MF and UF using porous (0.1 – 10 μm) and mesoporous (2 -10 nm) membranes, respectively, are often used in combination with biocatalysis for continuous production of valuable compounds and/or treatment of streams. Continuous membrane fermentors or cell recycle membrane bioreactors are applied when the reaction involves bacteria that perform the bioconversion during the growing phase and/or large size substrates that would not be able to enter the porous matrix (Chang et al., 1994; Giorno et al., 2002). In these cases, the membrane retains the biocatalyst and the large size substrate whilst it permeates the small size products. Examples of application of these systems include the production of carboxylic acids by fermentation of Lactobacillus bulgaricus (Choudhury & Swaminathan, 2006; Giorno et al., 2002). In cases where the bioconversion of large size substrate macromolecules is catalyzed by enzymes in order to retain it by MF or UF, it is necessary to enlarge its size, which is often obtained by immobilizing enzymes on nanoparticles (Chang, 2018). If the substrate is small enough to enter the membrane pores, then, the biocatalyst (bacteria in vegetative stage or enzymes) can be immobilized within porous matrices and the reaction occurs within the pore void volume (Giorno & Drioli, 2000; Giorno; et al., 2017). Examples of application of this configuration in biorefinery, include production of valuable compounds and energy vectors (Drioli & Giorno, 2009; Mazzei et al., 2013). The immobilization of enzyme in membranes demonstrated to increase enzyme stability

(Giorno & Drioli, 2000) without necessarily affecting the enzyme catalytic activity (Mazzei et al., 2012), supposed that the microenvironment is tuned to guarantee suitable enzyme macromolecular flexibility and rigidity, water activity (Vitola et al., 2017), substrate mass transport (Giorno et al., 2006).

NF (using membranes with 0.5 – 2 nm) is usually combined with biocatalysis carried out by free enzymes and it is used to fractionate small molecular weights intermediates (Tay et al., 2018). However, some example of enzyme immobilized on NF membranes was also reported (Dizge et al., 2018). Applications include fractionation of oligosaccharides, peptides, amino acids, organic acids.

MDSX is applied to carry out bioconversions using interfacial biocatalysts (such as lipases) immobilized within the membrane where the organic/water interface is also located (Giorno et al., 2007). Field of applications include production of active ingredients (Sakaki et al., 2001), processing of vegetable oils.

MD and FO are mainly used for concentration of biocatalyst or molecules upstream the membrane (Goh et al., 2015; Holloway et al., 2015; Song & Liu, 2019). This is usually the case when waters coming from agro-food industries are present in diluted streams that need to be concentrated in order to reduce processing costs. PV is used in combination to bioconversions to separate alcohols from water-based mixtures (Fan et al., 2016). ME is a relatively novel membrane process able to formulate emulsions on a drop-by-drop mechanism through the membrane pores, which disperse at high throughput, a non-miscible phase into another, at low energy input. ME was proven to be a powerful technique to assist bioconversion by separating reaction product (Mazzei et al., 2010) or by formulating biocatalysts distributed at the interface (Piacentini et al., 2021).

2. Use of MBRs in biorefineries

2.1 Cellulase and membrane processeses in biorefineries

The bioprocessing of agro-food residues, such as rice and wheat straws, sugar bagasse and corn stover with 30–50% of cellulose content, are under intense research and development, with promising results and high technological readiness levels (TRL). Cellulose enzymatic hydrolysis is considered one of the most costly steps in the bioconversion of lignocellulosic biomass (Malmali et al., 2015), which involves an interfacial heterogeneity of solid cellulose substrate and cellulase enzyme adsorption. Various studies confirmed that it is possible, via membrane technology, to retain the enzymes present in the system, while allowing the transfer of lower-molecular weight reaction products to pass through the membrane (Andrić et al., 2010a).

Table 2 is a comprehensive summary of these studies, and major points are discussed in more details below. Most of the cases utilize membranes with molecular weight 10-50 kDa cut-off i–(Table 2). Usually, the reaction mixture of the substrate and enzyme is recirculated in the membrane reactor, whereas a stream with the products is withdrawn from the permeate side. Flat sheet membranes in a side-stream configuration are prevalently used. Only in few systems, a submerged membrane hollow fiber configurations, which can be more beneficial in terms of fouling control, are used.

Major challenges that limits industrial scale MBRs for cellulose hydrolysis include low substrate concentration, enzyme microbial degradation, and membrane fouling. For example, the cellulose concentration (2-5w/v%) is considered low for industrial application as it leads to low glucose concentration in the permeate (Malmali et al., 2015; Nguyenhuynh et al., 2017).

174 *2.1.1 Discontinuous MBR and product inhibition*

175 During cellulose hydrolysis, although a 100% yield is expected due to enzyme
176 specificity, most batch reactions could not achieve this, due to enzyme product-
177 inhibition. The inhibition of cellulolytic enzymes by glucose, cellobiose (Berlin et al.,
178 2007), which are produced during saccharification (Cantarella et al., 2014; Ximenes et
179 al., 2011), released during lignocellulosic pretreatment, is a well-known problem. This
180 is exacerbated by the high enzyme cost, imparted by its dischargment and replacement.
181 The cellulase enzyme replacement contributes up to 20% of the total cost in case of
182 bioethanol production and ~50% of the entire hydrolysis step, limiting both the
183 technological and economic feasibility of the hydrolysis process. A detailed analysis of
184 the mechanisms and kinetics of the product-inhibition of cellulolytic enzymes by
185 glucose and cellobiose has confirmed that reactors should be designed with continuous
186 or semi-continuous product removal. As a result, numerous studies have focused on the
187 integration of membrane bioreactors (MBRs) in biorefineries for simultaneous
188 hydrolysis and continuous/intermittent *in-situ* product removal (Gebreyohannes et al.,
189 2013; Mahboubi et al., 2017b; Nguyen et al., 2015).

190 In this section we will discuss major research findings using intermittent/discontinuous
191 processes. A four-fold increase in enzymatic hydrolysis of cotton cellulose with
192 intermittent removal of the product cellobiose, by using a flat-sheet polyethersulfone
193 membrane was achieved (Gavlighi et al., 2013). Authors achieved 19% degree of
194 conversion after 3 days, for a reasonable feed concentration of 25 g/L.

195 The hydrolysis of microcrystalline pure cellulose powder was also evaluated in a
196 tubular MBR configuration and compared with a flat-sheet MBR (Bélafi-Bakó et al.,
197 2006). 95% of the cellulase was retained by membrane as estimated by dry weight

measurements and only 6% of the initial enzyme activity has been observed in the permeate. Thus, the membrane sufficiently retained both the substrate and enzyme. Possibly, due to better mass transfer, the tubular membrane gave 10% higher average conversion than the flat-sheet membrane configuration. In another MBR (Liu et al., 2011) configuration the cellulase from *Aspergillus niger* was free in solution and retained in the MBR by a polyethersulfone ultrafiltration membrane. Also in this system a complete retention of both cellulose and cellobiase was observed.

In a recent study, a modified submerged MBR for enzymatic cellulose hydrolysis was developed (Nguyenhuynh et al., 2017). In this work the intermittent product removal was used and in the mentioned conditions more effective UF performance with complete glucose permeation and enzyme retention up to 80% was obtained.

Qi et al. (Qi et al., 2012) examined the application of combined UF and NF for recovering the cellulase and concentrating glucose, respectively, in an integrated approach. They found that the UF membranes permitted a cellulase retention of 74%, a conversion of 84.5% and a recovery of all the glucose in the permeate.

Although UF based MBR was effective to retain the enzyme and limit enzyme product inhibition, the system was prone to membrane fouling. As a strategy to limit membrane fouling, Lim and Ghazali (2020) used an intermittent product removal during the continuous hydrolysis of microcystalline cellulose. The removal of the product from the bioreactor using UF membrane filtration was done under two different strategies. For Strategy 1, 50% of the reaction mixture was filtered after 4 h of hydrolysis reaction to remove the reducing sugar. The recycling of the enzyme and the filtration of the hydrolysate were carried out simultaneously. The hydrolysis reaction was continued and

the filtration was repeated at the 8th h. For Strategy 2, the fresh substrate and citrate buffer were added at a 24 h interval, while the filtration process started at the 24th h. Compared to the batch productivity (63% of cellulose conversion after 72 h), the intermittent product removal gave a 10x times higher productivity, due to the limited enzyme-product inhibition. The more frequent product removal, together with the enzyme recycling, was sufficient to main a reasonable reactor productivity. Table 2 also shows that most of the systems utilized side-stream MBR configuration, which enforces pumping a slurry. Recently, there is a growing effort and success in the use of submerged MBR in order to resolve this issue. A modified submerged MBR system with intermittent product removal developed recently for instance gave an effective UF performance with complete glucose permeation and up to 80% enzyme retention (Nguyenhuynh et al., 2017).

In another approach, the hydrolysis of α-cellulose was carried out in a submerged continuous MBR. Since an MF membrane was used in the submerged system, a pre-holding time was allowed in order to promote a better binding between enzyme and substrate (Malmali et al., 2015). The continuous hydrolysis with in-situ product removal gave an order of magnitude higher rate of glucose production relative to batch process, due to enzyme product-inhibition. On the other hand, the biocatalysis of carboxymethyl cellulose in an MBR equipped with MF and enzyme immobilized on magnetic nanoparticles led to a constant reaction rate over time, and 50% higher enzyme efficiency, due to in-situ product removal (Gebreyohannes et al., 2018). The use of biofunctionalized nanoparticles have the inherent issue of nanoparticle aggregation at high concentration. Hence, designing the system under reaction rate limited regime can prevent mass transfer resistance due to particle aggregation and the subsequent loss of

biocatalytic efficiency. In addition to *in-situ* product removal, the use of~~A~~ a cocktail of synergistically performing different cellulytic enzymes can be an effective strategy to reduce the extent of the enzyme-product inhibition (Gebreyohannes et al., 2018; Lozano et al., 2014). When the hydrolysis of carboxymethyl cellulose was run with a mixture of endoglucanase and β-glucosidase, in an MBR configuration higher monomer to oligomer ratio, was obtained due to absence of cellobiohydrolase and β-glucosidase inhibition by cellobiose and the and glucose, respectively (Gebreyohannes et al., 2018). Not only the use of mixture of these enzymes but also an appropriate ratio of cellulase and cellobiase is highly imperative to achieve rapid cellobiose hydrolysis and prevented the cellulase inhibition (Lozano et al., 2014).

2.1.2 Continuously fed MBR, limitation to low MWCO membrane and operational conditions

As shown in Table 2, most MBRs for cellulose hydrolysis are operated with a separated bioreactor and pumping of the slurry across the membrane for ultimate retention/recycling of the unreacted substrate and enzyme, while allowing permeation of glucose. In order to retain the 60 kDa cellulase enzyme (Suurnäkki et al., 2000), the membrane molecular weight cut-off used in this application is often limited to about 10 kDa (Giorno & Drioli, 2000; Tian et al., 2015). Andrić et al. (2010b) have previously indicated that an appropriate MBR design for continuous enzymatic hydrolysis with *in-situ* product removal is crucial. However, a side-stream configuration is a limiting factor to successful large scale applications, since pumping a slurry imparts a significant operating cost (Roche et al., 2009; Stickel et al., 2009). Moreover, low MWCO membranes require high transmembrane pressure and leads to significant membrane

fouling (Lim & Ghazali, 2020; Lozano et al., 2014; Mahboubi et al., 2017a). While a continuously fed MBR could face severe membrane fouling, owing to the enzyme retention and simultaneous product removal, a continuously/intermittently fed system can have better productivity.

For instance, a corn stover pre-treated by soaking in 15 wt% aqueous ammonia incubated with a cellulase loading of 60 FPU per initial cellulose was used to compare the performance difference among batch, continuously fed and intermittently fed MBR. Intermittent addition of 5 g/L cellulose every 8 h, gave a total glucose of 1.94 and 1.88 times higher than batch reactor without MBR and continuously fed MBR, respectively.

Yet, the obtained product concentration in many of the studies is considerably low (0.2-20 g/L,) (Gebreyohannes et al., 2018; Lim & Ghazali, 2020; Lozano et al., 2014; Zhang et al., 2011).–Since the desired concentration for subsequent fermentation to ethanol, falls between 150 to 250 g/L glucose (Malmali et al., 2015), a significant energy is consumed in pre-concentration. Increasing the substrate concentration specially when using high MWCO membrane can be one strategy to achieve a higher product concentration (Malmali et al., 2015).

In all these discussions, it was difficult to elucidate the contribution of the enzyme, as the type, amount and units of the enzymes used were different.

Various strategies have been employed to alleviate the issue of membrane fouling. A good example could be application of electro-ultrafiltration (EUF) during the filtration of pre-hydrolyzed acid pre-treated wheat straw to mitigate the membrane fouling. EUF is a method, where a differential electric field is applied across the membrane to achieve electrostatic repulsion of membrane foulants (Hakimhashemi et al., 2012). The flux

when the system was fed with 2% w/v lignocellulosic hydrolyzate increased by a factor of 4.4 at room temperature, compared to that without electric field

Moreover, intensification of the hydrolysis step with the fermentation process in a simultaneous saccharification and fermentation (SSF) seems to be the most promising strategy to increase overall productivity. The potential application of such hybridized system was recently shown by (Mahboubi et al., 2020).

The cellulose hydrolysis using MBR often requires low solid loading or low solid loading rate and continuous dilution in order to reduce the extent of membrane fouling, the enzyme product-inhibition and the difficulty of pumping a concentrated slurry. In order to resolve the issue of pumping slurry, a submerged MBR with a 10 kDa UF membrane was designed. Although the UF membrane was successful in retaining the enzyme (97%) and avoided pumping slurry, the cost for the pressurized reactor is considerable, while the membrane fouling was still severe (Zhang et al., 2011).

Alternatively, a submerged MBR integrating an MF membrane was employed (Malmali et al., 2015), which avoids pumping cellulose slurry. Owing to the use of MF, a high initial cellulose loading (100 and 150 g/L) was used, which are significantly higher than the cellulose loading observed in most MBRs (see Table 2). Higher substrate loading ensured higher glucose concentration; hence, the steady-state glucose concentration was 10-15 g/L. These values are significantly higher than the concentration obtained in the various UF systems. One of this systems' disadvantages is enzyme loss through the membrane. However, the extent of enzyme loss was limited by the introduction of pre-holding time that provided sufficient time for the enzyme to attach onto the cellulose. As a result, compared to the very high initial enzyme loading (50 mg/g cellulose), the rate of enzyme addition during continuous operation was either 4 or 10 mg enzyme/g

316 cellulose injected. In addition, the use of higher cellulose loading ensured more enzyme
317 retention.
318
319 *2.1.3 Biocatalytic membrane reactors in cellulose hydrolysis*
320 Commercial cellulase enzyme is often a cocktail of cellulolytic enzymes that include
321 endo/exo glucanase, cellobiohydrolase and β-glucosidase. However this mixture
322 generally exhibits low β-glucosidase activity (Rosgaard et al., 2006). Therefore, the
323 hydrolysis by endo-glucanase mainly favors the production of oligomers such as
324 cellobiose and cellotriose. As a result, Gebreyohannes, Dharmjeet (Gebreyohannes et
325 al., 2018) for instance obtained 50-60% higher oligomer productivity than monomers
326 when using an MF membrane system with immobilized enzyme. Over production of
327 cellobiose on the one hand causes enzyme product inhibition, while on the other hand it
328 may cause loss of significant amount of it to the permeate. In order to limit this
329 problem, it is imperative to supplement the system with additional β-glucosidase
330 (Andrić et al., 2010b). Especially co-immobilization of these enzymes in a biocatalytic
331 membrane reactor (BMR) configuration is highly beneficial. Accordingly, both
332 Gebreyohannes et al. (2018) and Song et al. (2016a) observed a significantly improved
333 monomer productivity by co-immobilization of cellulase and β-glucosidase in a BMR (4
334 times higher) and STR respectively. Enzyme immobilization is also a good strategy to
335 shift from UF membrane based MBRs to MF based BMRs that will eventually ensure a
336 higher volumetric reactor productivity.
337 For instance, the natural tendency of enzyme to be adsorbed by cellulose, often a
338 concern for enzyme efficiency loss, was taken as an advantage in order to retain the
339 enzyme in 0.6 µ MF equipped submerged MBR for cellulose hydrolysis.While this

system requires significant pre-holding time in order to ensure sufficient adsorption, the loss of enzyme is still unavoidable

In this case, membranes with immobilized enzyme in BMR configuration can be beneficial. Although the issue of enzyme leakage can be resolved through confining the enzyme on to the membrane or carrier particle, BMRs are less often used (Andrić et al., 2010a). However, since enzyme immobilization can contribute to the development of sustainable processes, it has substantial potential to be used in industrial lignocellulose-to-ethanol conversion. (Chang et al., 2011; Rodrigues et al., 2017)

A very recent strategy of biocatalytic systems is to immobilize enzymes on superparamagnetic nanoparticles (NP^{SP}). These particles afterwards are reversibly immobilized on a microporous membrane using an external magnetic field in a system named superparamagnetic biocatalytic membrane reactor (BMR^{SP}) (Gebreyohannes et al., 2015; Gebreyohannes et al., 2017). The immobilization of the enzyme on the NP^{SP} can improved stability, activity along with easy recovery using an external magnetic force. (Ladole et al., 2017; Lupoi & Smith, 2011; Song et al., 2016b; Xu et al., 2011) Due to the possibility of using MF membrane with immobilized enzyme, it was possible to achieve constant glucose productivity at high solid loading (2-10 wt% CMC), high solid loading rate (3-6 g/h) and negligible rate of fouling (0.008 bar/min) in a submerged system. This is an immense improvement of the lignocelluloisic hydrolysis, which is generally limited to UF membranes to retain the enzymes (Gebreyohannes et al., 2018).

On the basis of the reported studies on enzymatic cellulose hydrolysis, enzyme stability, enzyme turnover, membrane fouling and product concentration still remain open challenges. The reactor design must be fully considered, particularly to limit the enzyme

cost, which contributes 25-30% operational cost (Guo et al., 2018). Side-stream ~~The main~~ MBR configuration, which combines free enzyme carrying out the hydrolysis in bulk and a membrane that removes the reaction products, is by far the most investigated. In this configuration, the enzyme compartimentalization promoted by membrane process, guarantees enzyme re-use and product inhibiton limitation, showing huge potential in operational cost reduction. Since MF can only retain enzymes compartmentalized to membrane or carrier particles, it is less interesting to employ it in a side-stream configuration (Malmali et al., 2015). Over all, use of membrane was effective in retaining the enzyme and preventing enzyme-product inhibition through intermittent/continuous product removal. Though dictated by the frequency of feeding and product withdrawal, this strategy also helps to mitigate membrane fouling. In terms configuration, a hybridization of hydrolysis with fermentation could be a way forward towards industrialization. While a submerged MF equipped MBR with immobilized enzyme could be an optimal strategy to increase MBRs volumetric productivity.

2.2. β-glucosidase and membrane process in biorefinery

β-glucosidase is a key enzyme in determining efficiency of cellulase for biomass hydrolysis, but recently it has also gained attention for its ability to hydrolyze glycosidic substrates from vegetal biomass to produce aglyconic compounds, which have important therapeutic properties (Mazzei et al., 2012; Mazzei et al., 2009; Ranieri et al., 2018). The use of membrane bioreactors in the production of aglyconic compounds solved several problems: the continuous removal of the inhibiton product (glucose) from the reaction site, the extraction of the water unstable aglycones in organic solvents by multhiphasic MBR, (Mazzei et al., 2010) and the enzyme reuse. On the basis of the

problem treated (e.g. glucose inhibition, aglycones extraction, kinetic study etc), β-glucosidase was entrapped on polymeric membranes (Mazzei et al., 2012; Mazzei et al., 2009) or covalently attached on ceramic membrane (Fig 2A) (Mazzei et al., 2012)(Fig 2B)(Ranieri et al., 2018). By using both biocatalytic polymeric and ceramic membranes, it was possible to produce an intensified system, in which the production/extraction of the aglycone in a pure organic solvent was promoted (Fig. 2). In the mentioned system, the aglycone extraction process is obtained by recirculating a pure organic solvent, in which the compound is soluble, in the lumen of a tubular membrane. When the aqueous phase, coming from the biocatalytic membrane and containing the product, it reaches the membrane lumen, on the basis of the membrane emulsification process an unstable emulsion is produced, which permits the aglycone extraction from the aqueous to the organic phase (Mazzei et al., 2010)(Fig. 2 a and b). Due to membrane processes modularity, the intensified MBR/ME system with an MF/UF process (Conidi et al., 2014) or with two steps of membrane emulsification (Piacentini et al., 2019) was easily integrated (Fig.3). In the first work, olive mill waste water (OMWW) pre-treated by MF/UF steps and containing the glycosidic substrate (oleuropein) was fed to the intensified process, obtaining the same degree of conversion when pure substrate was used (Fig. 3A). In the second system, in addition to the production/extraction of oleuropein aglycone, its encapsulation in hydrophilic polymeric (Fig. 3B) or hydrophobic solid lipid particles (Fig. 3C) was also promoted (Piacentini et al., 2019).

Recently, a further improvement of the system in terms of conversion (93%) by using the enzyme free in solution and promoting aglycone extraction by ME process (Fig. 3D) was obtained (Mazzei et al., 2020). The role of the membrane, in this system, was to retain the enzyme and to wash out the glucose from the reaction mixture. This permitted

412 to re-use the biocatalyst for five consecutive reaction cycles, with no decay in
413 conversion. In the two last mentioned systems, olive leaves as source of biomass to
414 obtain the glycosidic substrate were used.
415
416 **2.3. Xylanase and MBR in biorefineries**
417 Xylan is the second most abundant renewable compound on earth and a sustainable
418 technology which permits the recovery/fractionation of xylo-oligosaccharides (XOS)
419 and monosaccharide from xylan is one of the current priorities in the research related to
420 biorefineries. On the basis of the type and content of substituents within the xylan
421 structure, the synergistic action of xylanase (in particular endo-1,4-β-xylanase and β-
422 xylosidase) and other debranching enzyme (α-L-arabinofuranosidases, α-
423 glucuronosidase, acetyl xylan esterases and ferulic acid esterases) is generally needed.
424 However, due to the product inhibition on the xylanases enzymes a separation step to
425 isolate the biocatalyst is necessary, particularly if a large scale and a continuous process
426 is needed.
427 A lot of recent articles propose membrane bioreactor technology to overcome the limits
428 given by product inhibition (Andrić et al., 2010a; Nabarlatz et al., 2007; Pinelo et al.,
429 2009; Sueb et al., 2017) and to simultaneously purify the product from the reaction
430 mixture.
431 However, it must be considered that the substrate tends to accumulate on the membrane
432 surface as gel-like aggregates, influencing the fluid-dynamic conditions and enzyme
433 kinetic properties.
434 In the work carried out by Sueb et al. (2017) the effect of fouling due to particle
435 deposition was evaluated by different configuration of MBRs. The MBRs configuration

used were: a) reaction (endo-1,4-b-xylanase and β-xylosidase, free state) and filtration (1 kDa PES membrane) in the same system; b) xylanase (free state) reaction and filtration in a MBR and a further enzymatic reaction of the permeate by xylosidase in a STR; c) both enzymes present in a stirred tank reactor and a subsequent filtration process. Reaction with both enzymes followed by UF (configuration C) was the optimal configuration, which permitted at least 40% higher xylan hydrolysis than the cascade configuration.

In the work carried out by Acosta-Fernández et al. (2020), a membrane with higher nominal molecular weight cut-off (10 kDa) was used starting from xylan from coffee parchment. In the mentioned research the enzyme free in solution or immobilized on magnetic nanoparticles, in 2 STRs and in 2 MBRs, were compared. Results demonstrated that by using the MBRs configurations a continuous production of xylooligosaccharides, with the molecular weight distribution in the range of prebiotic sugars (X1−X20) was obtained. By optimizing the fluid-dynamic conditions a high conversion can be also achieved at high substrate concentration. Besides, the unchanged apparent Km demonstrated that the enzyme immobilization procedure did not alter the affinity of the enzyme for the substrate and it was even improved when membrane process was present, since it promoted a continuous removal of inhibition products from the reaction mixture.

Biofunctionalized magnetic nanoparticles were also coupled to an organic-inorganic hybrid membrane (were magnetic nanoparticles were used as nanofillers) to develop a nano-inspired, magnetic-responsive enzyme membrane (micro) reactor (Gebreyohannes & Giorno, 2015). In this system xylanase and pectinase as model biocatalysts were used

to control membrane fouling. The system permitted 75% reduction in membrane filtration resistance through the membrane surface cleaning.

An integrated membrane process was also proposed by González-Muñoz et al. (2008), in which liquors containing xylan-derived products from rice husk was firstly treated with diafiltration and then by MBR to obtain and purify low molecular weight arabino-xylooligosaccharides (AXOS). Also in this study the various MBR configurations were studied.The best configuration in terms of productivity (93.3% recovery yield vs 75.8%) was the one in which the catalysis was carried out simultaneously with the separation process.

2.4. Pectinase and MBR in biorefineries

Pectin is a complex polymer of carbohydrates present in the cell wall of the main higher plants. In recent years, pectic biomass is considered as an important source of feedstock, because it contains a low lignin concentration and in some industrial process (e.g. juice filtration) is considered a waste material, which can be valorized through hydrolysis process. It can be also used as starting source to produce galacturonic acid, which is a raw material in food, pharmaceutical and cosmetic industry, due to its important properties or for pectin-derived oligosaccharides (POS). POS are an emerging class of prebiotic, but they can also have important therapeutic properties such as: ability to induce apoptosis in human colon cancer cells, anti-inflammatory and antiobesity properties, etc (Gómez et al., 2016). On the basis of the different pectic biomass used, oligosaccharides with different structure can be obtained such as arabinogalacto-oligosaccharides, arabinoxylooligosaccharides, galacto-oligosaccharides etc. Pectin hydrolysis can be carried out by both chemical and enzymatic methods, but as

483 frequently observed the enzymatic methodology offers several advantages such as
484 reaction in mild conditions avoiding corrosion, selective hydrolysis and higher reaction
485 yield. However the pectic enzymes generally suffer from product inhibition of the
486 monomer (galaturonic acid). For this reason, a separation process after hydrolysis is
487 highly desired. This is the reason why membrane processes are generally coupled with
488 enzymatic hydrolysis for pectin in MBR systems, which permit the continuous POS
489 production, enzyme re-use and conversion increase due to inhibition product
490 removal(Gómez et al., 2016). MBR technology for pectin hydrolysis is currently used
491 by both immobilized and non-immobilized enzyme, although the most used
492 configuration is with free enzyme recirculated in the retantate side (Table 3) (Alkorta et
493 al., 1995; Bélafi-Bakó et al., 2007; Rodriguez-Nogales et al., 2008; Rodríguez-Nogales
494 et al., 2005). In the last mentioned systems, both flat-sheet and hollow fiber membranes
495 made of different materials were used. Two kind of reactors are used: sequential batch
496 reactor and filtration (discontinuous) or simultaneous batch filtration process
497 (continuous). In the first case, the reaction occurs in a first step after a certain incubation
498 time without product separation. The membrane process is used in a second step to
499 carry out the purification. To avoid the excessive production of monosaccharides, small
500 amount of biocatalyst is used for this reason and the enzyme concentration to achieve
501 the highest conversion is one of the most studied parameters (Mountzouris et al., 2002;
502 Torras et al., 2008). The incubation time is another parameter frequently studied to
503 control the MW of the products, but the non-specific enzyme cleavage does not permit
504 to control it. As a result, batch reactors coupled with membrane processes are not
505 suitable for further application for the production of POS, since the final product have a
506 wide MW distribution (Moure et al., 2006). Strategies for final products separation are

507 based on the use of different membrane separation steps to obtain the different fractions
508 of the product. Córdova et al. (2017) used three different steps of nanofiltration for
509 oligosaccharides purification after hydrolysis in order to obtain products of target
510 properties grouped in the desired MW range.
511 Nevertheless, important viscosity reduction of pectin solution in the MBR with free
512 enzyme also without further purification by membrane processes is achieved, which is
513 very useful in systems in which a viscous solution must be treated (e.g. filtration of fruit
514 juice or olive mill waste water) and pectin causes membrane fouling (Gebreyohannes et
515 al., 2013). In the work carried out by Baldassarre et al. (2018), a discontinuous (used as
516 pre-treatment) and a continuous membrane reactor with free enzyme were used. This
517 permitted to increase the volumetric productivity up to five times, demonstrating a real
518 advantage respect to the traditional batch reactor. In the continuous MBR the process
519 was intensified, but the flow through the membrane was lower than discontinuous
520 systems, since large molecules tend to deposit on the membrane surface enhancing
521 transmembrane resistance. Nabarlatz et al. (2007) demonstrated that a high solute flux
522 during oligosaccarides fractionation caused an increase of concentration polarization
523 and an increased retention of low MW compounds. In particular a membrane selectivity
524 decrease (a broader range of oligosaccharides passed through the membrane) of about
525 25 % was observed when the flux was increased from 5 to 55 L $m^{-2}h^{-1}$.
526 Enzyme immobilization on membranes for POS production overcomes a lot of
527 problems related to both enzyme re-use and stability, targeted production of tailored
528 products, fast POS removal and hence limiting monomer production. Nevertheless, few
529 studies are currently applied for pectin hydrolysis in which BMRs are used. This can be
530 due to additional problems due to enzyme immobilization (steric hindrance, enzyme

aggregation) and/or enzyme deactivation due to chemical cleaning and disinfection of the biocatalytic membrane. Gebreyohannes et al. (2016) demonstrated that immobilizing the pectinase on magnetic nanoparticles, subsequently dispersed on the membrane surface by a magnetic field, permitted removal of the enzyme when necessary (e.g. membrane washing) and to distribute it avoiding steric hindrance improving enzyme kinetic performance. The use of biofunctionalized particles coupled with membrane process is widely employed now (Donato et al., 2012; Vitola et al., 2017; Vitola et al., 2019), since it permits to recover the catalyst at the end of the process, the possibility to clean the membrane with solvent without deactivating the enzyme and to keep unaltered the chemical-physical and morphological structure of the membrane, generally modified during chemical biofunctionalization.

2.5. Lipase and MBR in biorefineries

Membrane processes and in particular MBR are innovative systems for biodiesel production and can be used both in esterification, transesterification and biodiesel refining. The involvement of lipase in biorefineries is mainly in transesterification of tryalcylgricerides to produce fatty acid (m)ethyl esters (FA(M)EE). The enzymatic esterification process generally involves the presence of the lipase (free or immobilized) extracted from different microorganisms (*Pseudomonas fluorescens, Rhizopus Oryzae, Candida rugosa and Pseudomonas cepacia etc.*), an alcohol (ethanol or methanol) and a source of triglycerides, which could be vegetable oils, non-edible oils (e.g. Jatropha), waste cooking oil or animal greases, microalgal oil etc (Badenes et al., 2013). Compared to the chemical process, biological esterification is highly advantageous, since it promotes high conversion in mild operative conditions. Besides, in the

555 enzymatic transesterification, no soaps are produced, which imply the absence of further
556 washing steps, with the reduction of production costs and wastewater. The innovation of
557 MBR in the enzymatic esterification processes is also due to the process intensification
558 (reaction and separation in a single unit) which also significantly reduce the production
559 steps and the system compactness with respect to the traditional methods. However, the
560 enzyme cost is considered as one of the main limitation of MBR in general, which could
561 be reduced by the enzyme immobilization (Fjerbaek et al., 2009), because it
562 significantly increases enzyme stability and re-use. This is in fact the trend observed in
563 recent literature related to MBR and transesterification process (Table 4); where the
564 enzyme is almost always immobilized within polymeric membranes (mainly by
565 covalent attachment).
566 Another important problem to overcome in MBR is the enzyme deactivation due to the
567 interaction with methanol or ethanol. In particular, a molar ratio of methanol/oil higher
568 than 1/2 causes irreversible enzyme denaturation (Su et al., 2015)(Su et al., 2020).
569 Besides, the glycerol produced during the transesterification process, being more
570 soluble in water, limits the interaction of the enzyme with the substrate, forming a film
571 around the enzyme. This film does not permit the interaction with the hydrophobic
572 substrate, with a consecutive conversion decrease. To overcome this process, different
573 strategies were proposed, such as continuous addition of methanol, several methods for
574 methanol supply (in oil or in water), selective removal or glycerol etc. (Belafi-Bako et
575 al., 2002). Within the different strategies, the use of two-phase separated membrane
576 reactors, widely applied in MBR with lipase, seems one of the most promising
577 (Aghababaie et al., 2019). In the work carried out by Ko et al. (2012a), a two-phase
578 MBR permitted a stepwise addition of methanol and a selective removal of glycerol,

579 thanks to a regenerated UF membrane, coupled with a stirred tank reactor (STR). In this
580 case, the membrane role was to supply and remove methanol and glycerol respectively,
581 but it also worked as a contactor between the hydrophilic and hydrophobic phase (Fig.
582 4a). In the two-phase MBR developed by Aghababaie et al. (2019)(Fig. 4b) an
583 additional role of the membrane is to retain the biocatalyst, which is in the oil phase. In
584 both systems it was possible to reach a high conversion degree and stability.
585
586 **3. Challenges and future perspective on the use of MBR in biorefinery**
587 The main drawbacks which hindered the development of MBR in biorefinery industries
588 are mainly the low enzyme stability and the membrane fouling. To address these issues,
589 strategies also proposed in this review, must be taken into account, mainly related to the
590 selection of membrane material, operative conditions optimization and reactor
591 engineering design. In particular:

592 • the conjugation of biofunctionalized magnetic nanoparticle with membrane
593 processes can introduce an innovative strategy to selectively remove the
594 biocatalyst when fouling occurs. This will permit cyclic membrane cleaning
595 with solvents or backflushig, which are generally damaging for the enzyme.
596 • The use of estremophiles enzyme, which can tolerate high temperature could
597 alleviate cake-layer formation on the membrane, increasing the stability of the
598 biocatalytic membrane.
599 • The introduction of integrated membrane processes associated with MBR or
600 cascade enzymatic reactions in separated MBRs could be also interesting
601 strategies to pre-treat the stream before the enzymatic reaction, permitting
602 membrane foulig and enzyme reaction to be checked in separated steps.

- Another interesting approach is the possible use of microfiltration membranes with immobilized enzyme in a submerged configuration, which can ensure large volumetric productivity.

In order to fully apply the mentioned strategies in future applications, the integration between membrane science, genetic engineering, and chemical engineering is needed.

4. Conclusions

The use of MBRs in biorefineries for the first time was critically analyzed. Carbohydrate hydrolysis, biodiesel production, aglycones production, POS and galacturonic acid production and XOS production were described and critically reviewed.

In all the analysed sectors MBRs promote continuous reaction system, enzyme re-use and removal of inhibiting products, while increasing the system efficiency. To promote the development of MBRs on a larger scale some drawbacks (of this technology must be considered. Innovative strategies proposed in this review, can promote advances in membrane saving, membrane fouling control and enzyme stability improvement.

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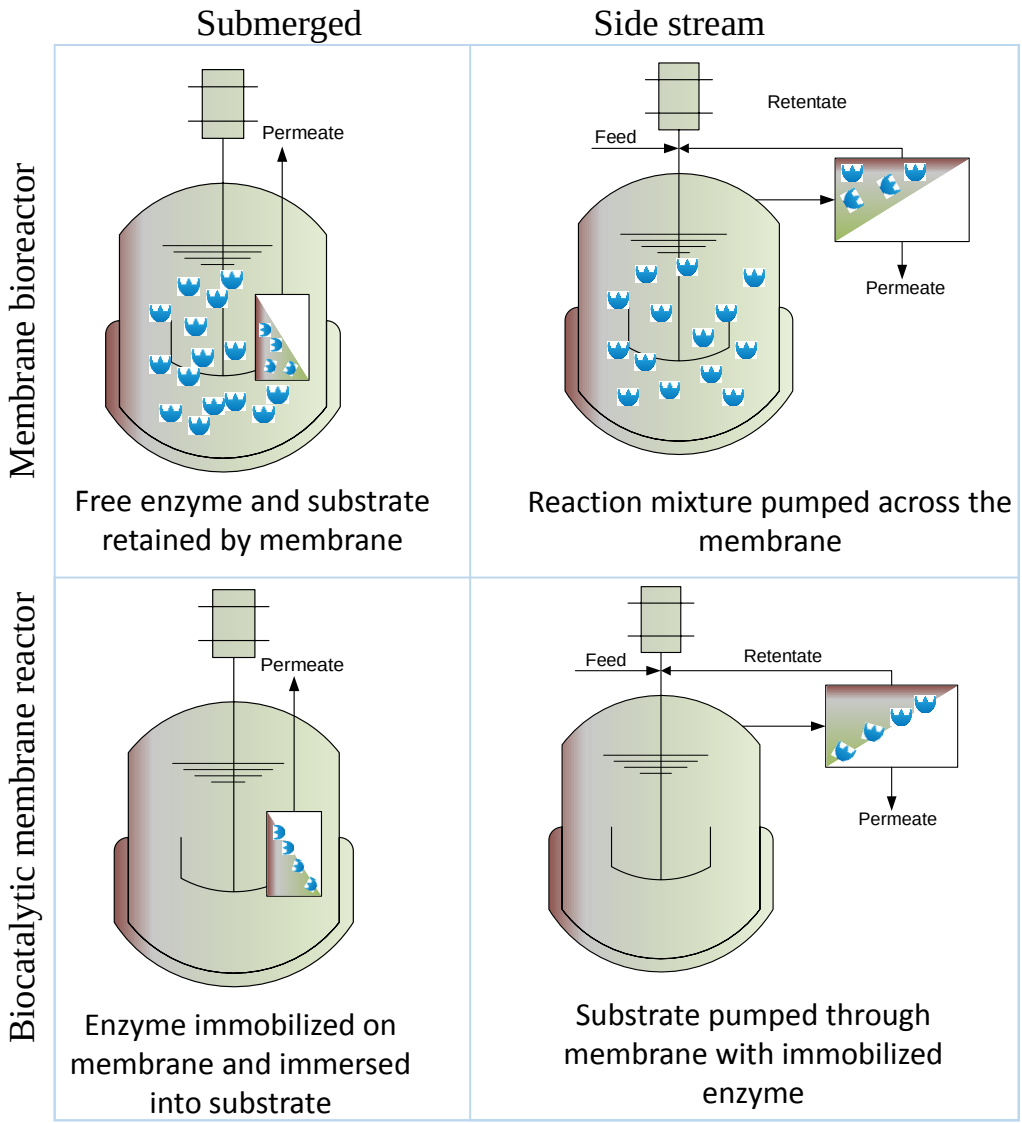


Fig. 1 Schematic representation of membrane bioreactor (MBR) and biocatalytic membrane reactor (BMR) in side-stream and submerged configuration. In the MBR the enzyme is free, while in the BMR the enzyme is immobilized.

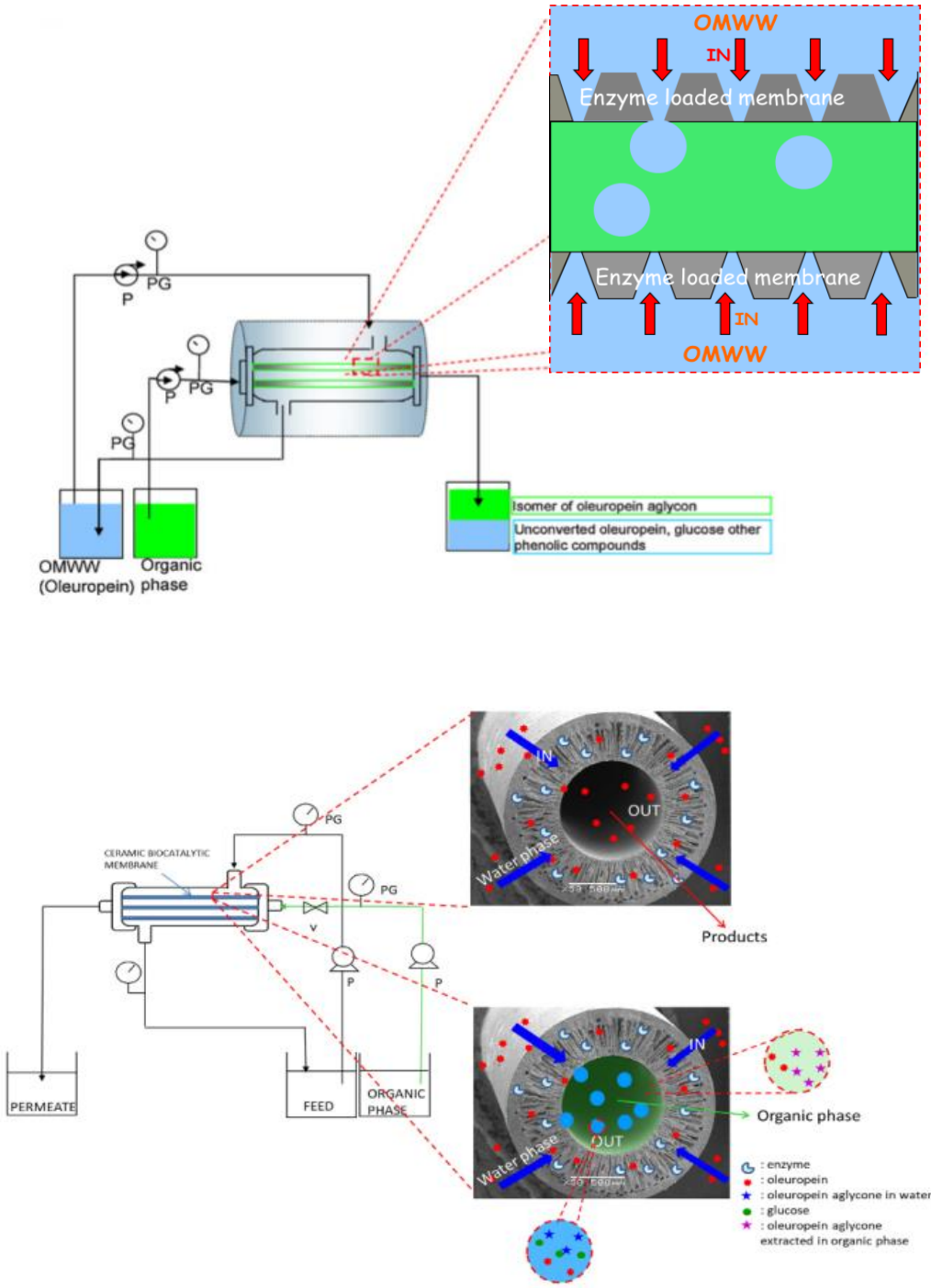
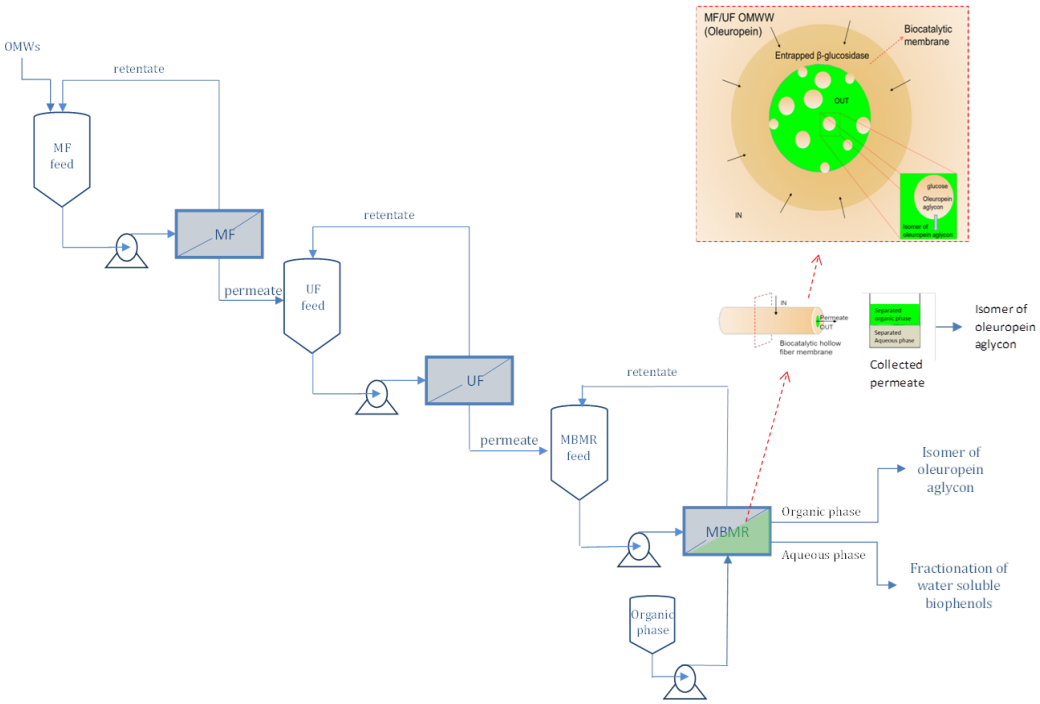
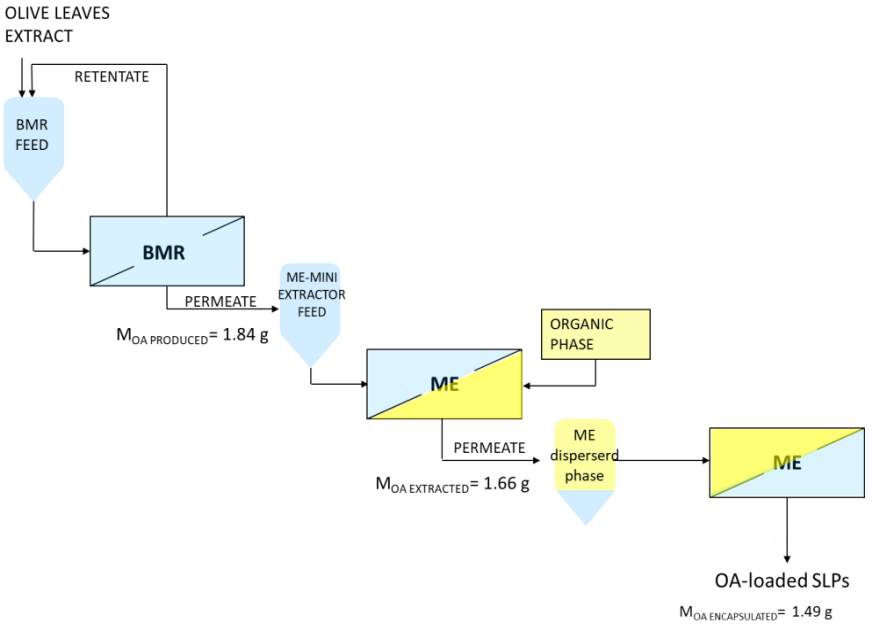


Fig. 2 Intensified membrane processes, in which MBR and membrane emulsification were coupled in a multhipashic system to promote production/extraction (in organic solvent) of aglycone. A: use of commercial polymeric membrane and physical enzyme immobilization, adapted from (Mazzei et al., 2012) with the permission of Copyright (2021) Elsevier; B: use of home-made ceramic membranes and covalent enzyme immobilization reprinted with permission from (Ranieri et al., 2018) with the permission of Copyright (2021).OA: oleuropein aglycone (product of oleuropein hydrolysis by β-glucosidase action), OMWW: olive mill waste water

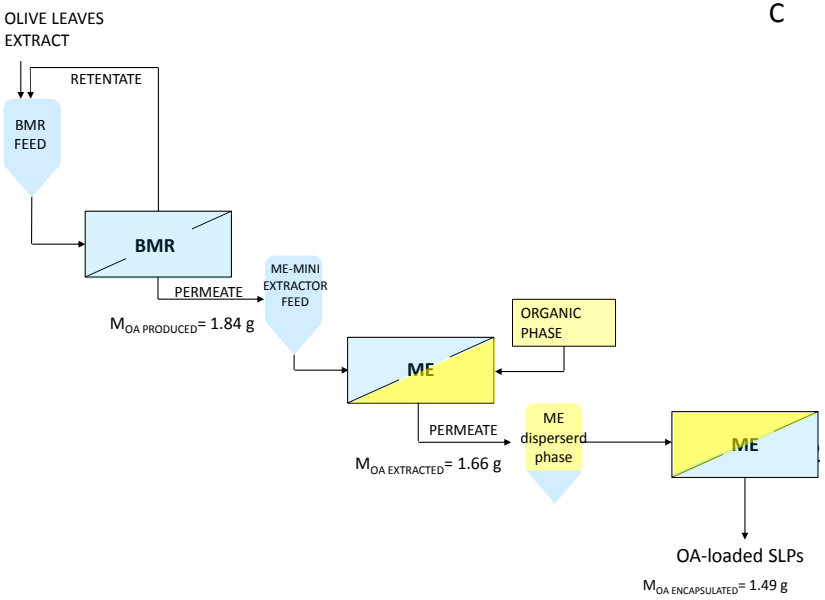
A


OMWs
retentate
MF feed
MF
permeate
UF feed
retentate
UF
permeate
MBMR feed
retentate
MBMR
Organic phase
Aqueous phase
Isomer of oleuropein aglycon
Fractionation of water soluble biophenols
Organic phase
MF/UF OMWW (Oleuropein)
Entrapped β-glucosidase
Biocatalytic membrane
OUT
IN
Biocatalytic hollow fiber membrane
Collected permeate
Isomer of oleuropein aglycon

B


OLIVE LEAVES EXTRACT
RETENTATE
BMR FEED
BMR
PERMEATE
$M_{OA\ PRODUCED}$= 1.84 g
ME-MINI EXTRACTOR FEED
ORGANIC PHASE
ME
PERMEATE
$M_{OA\ EXTRACTED}$= 1.66 g
ME dispersed phase
ME
OA-loaded SLPs
$M_{OA\ ENCAPSULATED}$= 1.49 g

C


OLIVE LEAVES EXTRACT
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ME-MINI EXTRACTOR FEED
ORGANIC PHASE
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ME dispersed phase
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OA-loaded SLPs
$M_{OA\ ENCAPSULATED}$= 1.49 g

D

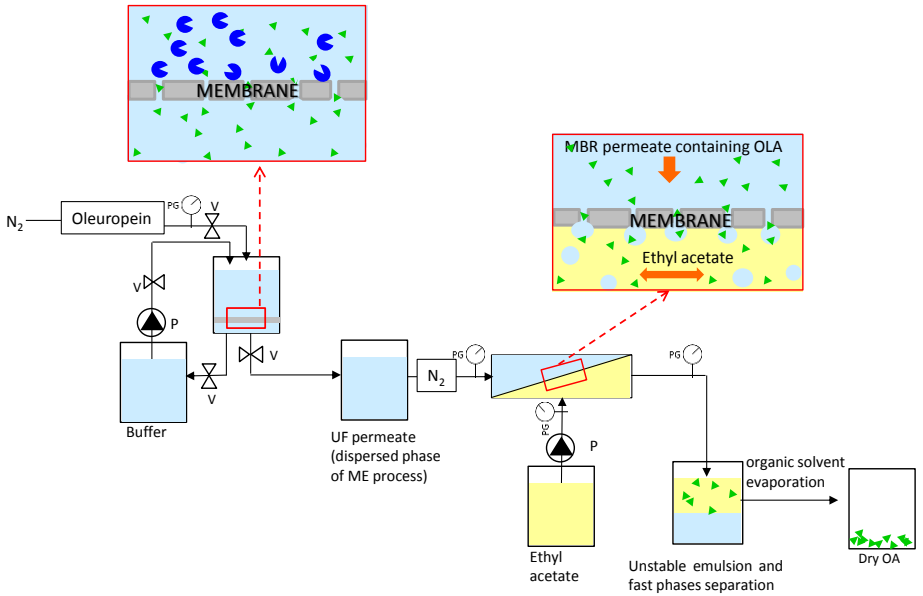
MEMBRANE
MBR permeate containing OLA
MEMBRANE
Ethyl acetate
N_2
Oleuropein
PG
V
P
Buffer
UF permeate (dispersed phase of ME process)
N_2
PG
P
Ethyl acetate
organic solvent evaporation
Dry OA
Unstable emulsion and fast phases separation

Fig. 3 Multhiphasic membrane bioreactor integrated with different membrane processes for the production of aglycone or formulated aglycone starting from different biomass. A) MBR integration with MF and UF process starting from olive mill waste (OMW). Reprinted with permission from (Conidi et al., 2014). Copyright (2021) Elsevier; B) MBR integration with two steps of membrane emulsification processes to produce solid lipid particles (SLP) containing oleuropein aglycone, starting from olive leaves. Reprinted with permission from (Piacentini et al., 2019). Copyright (2021) American Chemical Society; C) MBR integration with membrane emulsification processes to produce PVA particles containing oleuropein aglycone starting from olive leaves; Reprinted with permission from (Piacentini et al., 2019). Copyright (2021) American Chemical Society D) Integration of MBR with membrane emulsification process to produce aglycone from olive leaves reprinted from (Mazzei et al 2020)(CC-BY 4.0 licence). OA: oleuropein aglycone (product of oleuropein hydrolysis by β-glucosidase action)

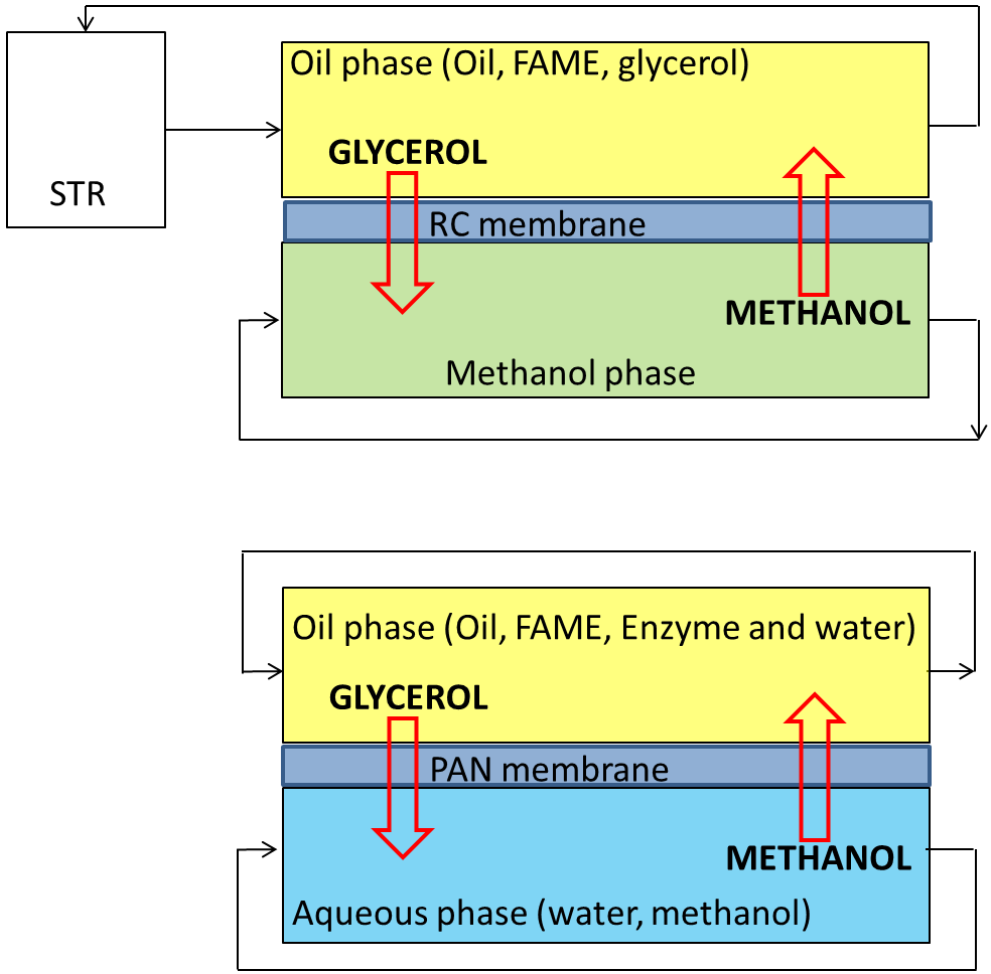

Fig. 4 Scheme of glycerol removal and methanol supply to two-phase MBR. A) system developed by Ko et al. (Ko et al., 2012b); B) system developed by Aghababaie et al. (Aghababaie et al., 2019).

Table 1 Membranes and membrane reactors in combination with enzymes in biorefinery.

Type of membrane	Membrane process	Role of membrane	Biocatalyst form	Type of Reactor	Ref.
Porous, hydrophilic	MF	Retain /recycle biocatalyst (microorganism, enzyme). Clarify stream	Free bacteria	Cell-recycle Membrane Bio-Reactor (MBR)	(Choudhury & Swaminathan, 2006; Giorno et al., 2002)
			Enzyme immobilized on particles	Enzyme-loaded-particles recycle MBR	(Chang, 2018)
			Enzyme immobilized on membrane	Biocatalytic Membrane Reactor (BMR)	(Giorno & Drioli, 2000; Giorno & Drioli, 2009; Giorno; et al., 2017; Mazzei et al., 2017a; Mazzei et al., 2013)
Mesoporous, hydrophilic	UF	Retain / recycle biocatalyst Remove inhibitors, products	Free enzyme	Enzyme-recycle MBR	(Giorno; et al., 2017) (Drioli & Giorno, 2009; Mazzei et al., 2013) (Vitola et al., 2017)
			Immobilized enzyme	Enzyme-loaded BMR	(Giorno & Drioli, 2000; Giorno; et al., 2017) (Drioli & Giorno, 2009; Mazzei et al., 2013) (Giorno et al., 2006) (Vitola et al., 2017)
Microporous , hydrophilic	NF	Fractionate, separate small molecular weight molecules	Free enzyme, immobilized enzyme	Enzyme-recycle MBR	(Chon et al., 2012)
			Immobilized enzyme	Enzyme-loaded BMR	(Dizge et al., 2018)
Porous, mesoporous, hydrophilic,	MBSX	Assist/implement interfacial reactions in biphasic systems.	Immobilized enzyme	Enzyme-loaded BMR	(Giorno et al., 2007; Sakaki et al., 2001)

hydrophobic		Extract molecules			
Porous, hydrophobic	MD	Concentrate molecules	Free bacteria	Cell-recycle MBR	(Goh et al., 2015)
			Free enzyme	Enzyme-recycle MBR	
Dense, hydrophilic	Forward Osmosis (FO)	Concentrate molecules	Free bacteria	Cell-recycle MBR	(Holloway et al., 2015; Song & Liu, 2019)
			Free enzyme	Enzyme-recycle MBR	
Dense, Hydrophilic	Pervaporation (PV)	Separate product, remove water	Free bacteria	Cell-recycle MBR	(Fan et al., 2016)
			Free enzyme	Enzyme-recycle MBR	
			Free enzyme	Enzyme-recycle MBR	
Porous, hydrophilic, hydrophobic	Membrane Emulsification (ME)	Enzyme distribution at O/W or W/O interface on droplets/particles surface	Immobilized enzyme	Enzyme-loaded-particles recycle MBR	(Mazzei et al., 2010; Piacentini et al., 2021)
		Solvent extraction via high throughput droplets formation		Enzyme-loaded BMR	

Table 2. Enzymatic hydrolysis of cellulose in MBRs.

Enzyme source	Enzyme content	Membrane			Feed	Conversion (%)/glucose mM	Feed concentration	Product concentration	Ref.
		Material[a]	Type[b]	MWCO (kDa)					
Trichoderma reesei	n.d.	polymeric	HF FS	n.d. 30	Microcryst. cellulose powder	48-53	2.5% (w/v)	3.7-6.5 g /h dm^3	(Bélafi-Bakó et al., 2006)
Trichoderma reesei	n.d.	PES	FS	10	Oil palm empty fruit bunch	n.d.	20 g/L	2-4 g/L	(Ghazali et al., 2017)
Aspergillus niger	1.5 g/L	PES	FS	10	Sodium carboxy methyl cellulose	40-90	1.5 g/L	1.2 g/L	(Liu et al., 2011)
Trichoderma reesei	n.d	PES	FS	10	Microcryst. cellulose	80	5-20 g/L	4.4-12.2 g/L	(Lim & Ghazali, 2020a)
Trichoderma reesei	1.36 g/L	PES	FS	10	Microcrystalline cellulose	80	10 g/L	5.48-6.45 g/L	(Lim & Ghazali, 2020b)
Cellic Ctec2	n.d.	PES	FS	0.3 μm	Dilute-acid pretreated wheat straw	70-80%	14.0 g/L	14.65 ± 0.59 g/L	(Mahboubi et al., 2020)
n.d.	3% w/w enzyme to substrate ratio	PES	FS	10	Microcryst. cellulose	n.d.	10% w/v	7.6 g/L	(Nguyenhuynh et al., 2017)
n.d.	0.7 g/l of α-amylase and 0.42 g/l of amyloglucosidase	PDMS/PET/PI	FS	n.d.	Broomcorn seed flour	n.d.	45 g/l	25.5 g/L	(Farahi et al., 2018)
n.d.	0.5 g/L	PES	FS	10	α-cellulose	45	10 g/L	2-8 g/L	(Abels et al., 2013)
Trichoderma reesei	4 g/L	ZrO_2	FS	10	Olive mill solid residue	45	n.d.	2-11 g/L	(Mameri et al., 2000)
n.d.	20 FPU/g cellulose	PES	FS	5 10	Steam exploded wheat straw	84.5	10% w/v	26.5-30.4 g/L	(Qi et al., 2012)

				30					
Trichoderma reesei	20 to 80mg/g substrate	PES	FS	5	Waste paper	67.4	20-100 g/L	12-50 g/L	(Rad et al., 2017)
Trichoderma reesei	20 FPU/g substrate	PS	HF	10	Steam-exploded rice straw	n.d.	125-185 g/L	15-35 g/L	(Yang et al., 2006)
Trichoderma longibrachiatum	*20 FPU/g dry mass*	PES	FS	-DF20 - 10	acid treated wheat straw	50.3 (%)	0.5-10%	n.d.	(Chen et al., 2013)
Crude cellulase powder		PS	HF	30	CO_2 laser treated corn Stover	-			(Chen et al., 2013)[c]
Trichoderma reesei		PC	-	/0.22 µm	carboxy methyl cellulose (CMC)	54 (%)	20 g/L	0.5-2 g/L	(Nguyen et al., 2015)
Novozyme cellulase	317.24 mg proteins/mL	-	-	10, 20	carboxy methyl cellulose (CMC)	1-	2.5 g/L	n.d.	(Cantarella et 2014)
Trichoderma reesei		PS		10	Pretreatd corn stover	(continuous)	15 g/L	10-30 g/L	(Zhang et al., 2011)
Trichoderma reesei		PVDF	FS	0.2 µm	Carboxymethyl cellulose	/0.9	0.5 wt%	90-160 mg/L	(Gebreyohannes et al., 2018)
Cellic CTec2		PES	FS	0.62 µm	α-cellulose	/0.08-0.11	100-150 g/L	40-100 g/L	(Malmali et al., 2015)
Trichoderma reesei niger		PES	TUBULAR	10	microcrystalline cellulose	/113	0.8 -2 w/v %	19.8 g/L	(Lozano et al., 2014)

[a] PES: polyethersulfone; PAN: polyacrylonitrile; PA: polyamide; PS: polysulfone; PC: polycarbonate.
[b] FS, flat-sheet; HF, hollow fiber
n.d., no data available in most cases, pH 4.8-5.0 and temperature 40-50^{o}C

Table 3 Use of MBR in pectin hydrolysis.

Pectin source	Enzyme	Enzyme status	Product/work aim	Membrane cut-off (kDa)/pore size (µm)	Membrane material	Reference
Apple pomace	Endopectidase, polygalacturonase	F	fouling control	10	PS	(Rodriguez-Nogales et al., 2008)
Sugar beet, black currant, red currant	Polygalacturonase from *Aspergillus niger*	F	galacturonic acid/	45/	PES	(Kiss et al., 2009)
Commercial pectin	Polygalacturonase from *Aspergillus niger*	F	galacturonic acid/ study of enzyme inhibition	30/	RC	(Bélafi-Bakó et al., 2007)
Onion skin	Viscozyme (mixture of enzymes)	F	POS/	10/	PS	(Baldassarre et al., 2018)
Lemon peels	Pectinex Ultra SP-L, pectinases from *Aspergillus aculeatus* and Pectinase 62 L	F	POS/	1/	RC	(Gómez et al., 2016)
Sugar beet	Viscozyme L,	F	POS/	10/	PS	(Elst et al., 2018)
Citrus pectin	Polygalacturonase from A.niger	IMM	POS/	/0.05−0.1	titania	(Szaniawski & Spencer, 1996)
Olive mill waste water	pectinex 3XL	IMM	/pectin hydrolysis	/0.4	PE	(Gebreyohannes et al., 2013)
Citrus fruit pectin	polygalacturonase	IMM	/membrane fouling	/0.1	PVDF	(Gebreyohannes et al., 2016)

PS: polysulphone, PES: polyethersulphone, RC: regenerated cellulose, PE: polyethylene, PVDF: polyvinylidene fluoride, IMM: immobilized, F: free

Table 4 MBR systems for bioediesel production.

Enzyme	Enzyme status /Immobilization	Membrane	Membrane (kDa)/pore size (μm)	TAG source	Alcohol	Conversion (%)	Stability (days)	Ref.
Lipase from *Candida sp. 99–125*	IMM/ adsorption	textile	-	salad oil and waste oil	MeOH in n-hexane	96	more than 20	(Nie et al., 2006)
Lipase from *Candida sp.* 99–125	IMM/ covalent	textile	-	lard	MeOH	85	7.5	(Lu et al., 2007)
Lipase from *P. fluorescens*	IMM/ adsorption	PES	300/	triolein	MeOH	80	12	(Machsun et al., 2010)
Lipase from *P. fluorescens*	IMM/ covalent	PVDF	/0.45	soybean oil	MeOH in n-hexane	95	7	(Kuo et al., 2013)
Lipase from *P. cepacea*	IMM/ covalent	PAN	-	soybean oil	MeOH	90	10	(Li et al., 2019)
Lipase B form *C. antarctica* l (CalB)	IMM/ covalent	RC	10, 25, 50/	soybean oil	MeOH	97.5	-	(Ko et al., 2012b)
Lipase from *C. rugosa* (Amano AY-30)	IMM/ covalent	PVDF	/0.45	soybean oil	MeOH	97 and 95,	7	(Kuo et al., 2013)
Lipase from *Mucor miehei*	IMM/ covalent	PES	/0.65	sunflower seeds oil	Bu-OH	100	missing data	(Handayani et al., 2016)
Lipase from *C.rugosa*	F/-	PAN	100/	*Eruca sativa* oil.	MeOH	100	3	(Aghababaie et al., 2019)
Lipase B from *C. antarctica*	IMM/ covalent	PAN	-	soybean oil	MeOH	80	12.5	(Li et al., 2019)
Lipase from *T. lanuginosus*	F/-	PAN	/0.2	Sunflower oil	MeOH	-	-	(Sokač et al. 2020
Lipase	IMM	PES	/0.001	Karanja oil	EtOH	88	-	(Kumar 2021

PES: polyethersulphone: PVDF: polyvinylidene fluoride, PAN: polyacrylonitrile, RC: regenerated cellulose, IMM: immobilized, F: free, MeOH: methanol

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: