

**Evaluation of pectin-reinforced supported liquid membranes containing
carbonic anhydrase: The role of ionic liquid on enzyme stability and CO₂
separation performance**

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Abstract

In this paper, pectin-reinforced, supported liquid membranes (SLMs) prepared with carbonic anhydrase (CA) were investigated for CO₂/N₂ separation. In the first part of the study, the effect of [Bmim][NTf₂] ionic liquid (IL) – as possible solvent to fill the pores of cellulose acetate support during SLM fabrication – on enzyme activity was tested. It turned out that this particular IL caused rapid and severe loss of initial biocatalyst activity, which fact can be seen as a threat in the membrane process design. Afterwards, the stability of pectin-containing SLMs (containing CA but lacking the IL having adverse impact) was addressed and their improved resistance against higher transmembrane pressures (up to 7.2 bar) was found, representing an approx. 3-fold enhancement compared to their control. Thereafter, the performance of the membranes was tested under single and mixed gas conditions with carbon dioxide and nitrogen. Employing single gases, it was demonstrated that CA enzyme could notably increase CO₂ permeability (from 55 to 93 Barrer), while that of N₂ remained unchanged (1.6-1.7 Barrer). Thus, the highest CO₂/N₂ theoretical selectivity was attained as 54 using the pectin-reinforced SLMs enriched with CA biocatalyst. For comparison, the outcomes were plotted on the Robeson upper-bound.

Keywords: gas separation; supported liquid membrane; ionic liquid; carbonic anhydrase; CO₂ separation

1. Introduction

The enhancement of CO₂ separation from various gaseous mixtures (including flue-, bio- as well as natural gas) via the design of novel, facilitated-transport membranes has become a topic of wide interest [1]. Improved CO₂-permeation capability in these types of membranes can be achieved in several different ways [2], where popular methods cover the incorporation of membrane materials such as polymers with specific chemical agents/solvents and in recent year, membrane preparation by using enzymes, in particular carbonic anhydrase (CA) has drawn attention too. This latter, biocatalytic route – that transfers carbon dioxide via a reversible reaction to form bicarbonate as introduced in our previous paper [15] – has been emphasized as a possible way forward in advancing new-generation carbon dioxide capture technologies, which are less energy-intensive, show faster reaction kinetics [3] and provides membranes with better permselectivity. The separated CO₂ can be used for the synthesis of valuable components [4] such as organic acids [5], energy carrier e.g. methane [6]. Further utilization path of CO₂ may involve algae cultivation [7], intensification of anaerobic hydrogen fermentation [8], etc.

So far, the CA enzyme has been applied with success in different membranes applications. Relevant examples by Hou et al. [9,10], Yong et al. [11] proved that CA or its mimicking substance i.e. Zn-cyclen [12] can fit to upgrade gas-liquid membrane contactors and membrane reactors [13]. In another research direction, supported liquid membrane (SLM) prepared with

the addition of CA was found as a feasible approach in membrane development [14-17]. Conventional SLMs are fabricated by filling various sorption liquids to the pores of polymer membranes.

Among SLMs, those made with solvent e.g. ionic liquids (IL) are regarded as supported ionic liquid membranes (SILMs) and represent an emerging class for gas separation purposes [18-21]. Though SILMs are promising from many aspects, issues related to their mechanical stability due to the removal of ILs from the pores at relatively low transmembrane pressure differences may occur. To overcome such liquid washout and consequent membrane degradation, solutions such as membrane gelation (achieved via the blending of ILs with polymers) have been tested [22]. As gelling material, the group of Coelho [22,23] applied gelatin, which is a cheap and widely available biopolymer. This example is a good indication of the potential that naturally-occurring components can have in SILM development.

In addition to membrane integrity, the biocompatibility of ILs should be of concern too, as it may significantly affect longer-term activity of enzyme mixed and immobilized in it [24]. In fact, Martins et al. [16] have also underlined that biocompatible and environmental-friendly ILs can be favored for SILM synthesis. It was noted in previous works that small quantities of CA enzyme (0.1 mg/g IL) [16,23], even in partly-purified form after recovering it from biomass [15] can work and effectively shuttle CO₂ across the SILM membrane. However, to our knowledge, the time-dependent change of CA activity in ILs has not been monitored so far.

Given that SILM durability can be influenced by the above-referred structural and biological impacts, the aim of this study were two-folded. Firstly, we have assessed the IL-CA interactions as a crucial parameter of membrane lifetime employing [Bmim][NTf₂], which was used for the preparation of enzymatically-boosted SILMs in our previous investigation [15]. Secondly, CA-containing membranes gelated with pectin – a natural biopolymer found in plants [25] – were evaluated against pressure-resistance, followed by gas permeation tests carried out with pure (CO₂, N₂) and mixed (CO₂ – N₂) gases.

As far as we know, this is the first report on the behavior and use of CA-enriched, pectin-containing membranes for CO₂ separation and hence, the information delivered can be novel enough and helpful for the international research community of membraneologists.

2. Materials and methods

2.1. Enzyme and chemicals

Throughout the experiments, the CA enzyme purchased from Sigma-Aldrich, USA – product ID: C2624, purity: >95 %, specific activity: >3500 Wilbur-Anderson (W-A) unit mg⁻¹ protein – was used. The ionic liquid, 1-Butyl-3-methylimidazolium bis(trifluoromethylsulfonyl)imide ([Bmim][NTf₂], purity: >99 %) was obtained from Io-Li-Tec, Germany. Pectin (type: Pectin Amid CU 025; degree of esterification and amidation is 29 % and 23 %, respectively; galacturonic acid content: 89 % according to the certificate of analysis

provided by the manufacturer) was ordered from Herbstreith & Fox KG, Germany. Although a huge variety of pectin is available on the market, this one was specifically chosen for the experiments since it does not contain sugars, which can be considered as an advantageous property from the microbiological stability viewpoint of the gels prepared with it. $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$ was the product of Sigma-Aldrich, USA.

2.2. Enzyme activity assays

Basic procedure. The activity of CA (EC number: 232-576-6) was determined in W-A unit mg^{-1} enzyme. To conduct the measurements, a stock enzyme solution (SES) (2 mg CA mL^{-1}) had to be first prepared using Tris-HCl buffer (0.02 M , $\text{pH} = 8.3$). Thereafter, $20 \text{ }\mu\text{L}$ SES was diluted (D-SES) to 10 mL with Tris-HCl buffer (0.02 M , $\text{pH} = 8.3$). Afterwards, 14 mL Tris-HCl buffer (0.02 M , $\text{pH} = 8.3$) was mixed with 1 mL D-SES in a reaction vessel (thermostated to $0 \text{ }^\circ\text{C}$) and 6 mL substrate solution (CO_2 -saturated distilled water) was added simultaneously. The whole container was continuously stirred at 450 rpm with magnetic bar. Once the reaction mixture was complete, the time needed for 1 unit of pH fall (in the range of $8.2\text{-}7.2$) was measured by stopwatch. Complementary tests were also performed under enzyme-less circumstances. The W-A unit was delivered from the times elapsed under the two conditions (with and without CA enzyme) according to the formula

introduced in our previous paper [15]. This was then normalized by the mass of enzyme in the reaction mixture to get the values in W-A unit mg^{-1} enzyme.

Modified procedure I. The *Basic procedure* was adopted with some alterations to check CA activity in the membranes prepared. The membranes were cut to 4 x 4 mm pieces, some of which was placed to the reaction vessel together with 15 mL Tris-HCl buffer (0.02 M, pH = 8.3) and 6 mL substrate solution.

Modified procedure II. The *Basic procedure* was adopted with some changes to reveal the effect of [Bmim][NTf₂] ionic liquid on the CA enzyme activity. During these experiments, 9 mL [Bmim][NTf₂] ionic liquid was mixed with 1 mL SES, giving a mixture referred as IL-SES. Next, the enzyme activity was measured every 5 minutes for a couple of cycles. To do so, 3 mL of the IL-SES was transferred to 12 mL Tris-HCl buffer (0.02 M, pH = 8.3), supplemented with 6 mL substrate solution and the time required for 1 unit of pH drop (from 8.2 to 7.2) was recorded in order to compute the corresponding W-A unit mg^{-1} enzyme, as mentioned before. Additional test were run under enzyme-less circumstances.

2.3. Membrane preparation

Porous, hydrophilic, cellulose acetate membrane (pore size: 0.2 μm , porosity: 60 %, thickness: 120 μm , Sartorius AG) with 5.6 cm diameter was placed to a Petri-plate and then it was moved to a vacuum desiccator for 30

minutes. This was followed by two consecutive steps: (i) filling 2 mL SES to the membrane surface/pores and (ii) 30 minutes of vacuum again. As the time expired, a mixture of 4 mL pectin solution (0.25 wt%) and 140 μ L CaCl_2 solution (1 wt%) was distributed as equally as possible on the surface of the membrane. Another 30 minutes was allowed to achieve partial gelation. In the last stage, the membrane was taken out of the desiccator and forced between 2 glass panes to (i) remove excess pectin that did not strongly bind to the membrane pores and (ii) finish the gelation process.

Afterwards, activity, stability and gas permeation tests on the membranes could be performed. Besides these membranes containing the CA, additional ones lacking the enzyme were made too for comparison. Based on weighing, the reinforcement by pectin resulted in an average gain of of 400-500 mg (on wet basis) for the freshly made membranes. Furthermore, the thickness of the pectin/cellulose acetate membranes was $160 \pm 30 \mu\text{m}$.

2.4. Gas permeation device

The gas permeation experiments were carried out in a two-chamber permeation apparatus, including a permeation cell that hosts the membrane [19].

In the course of single gas tests, both (the feed and permeate) chambers of the permeation cell were purged with the given gas, followed by setting the pressure on the feed and retentate sides to 1.7 bar(a) and 1 bar(a),

respectively. Similar driving force (~ 0.7 bar) was applied by [Neves et al. \[26\]](#), as well.

Under these conditions, once the chambers were closed, the gas started to pass from the higher pressure to the lower pressure compartment. This progress (pressure equalization) was monitored by pressure transducers on both sides as the function of time by in LabVIEW. A typical time profile of the permeation experiments is displayed in **Fig. 1**. The (pressure vs. time) data were first processed by the methodology described in the paper of [Neves et al. \[17\]](#), Afterwards, the permeability (p) of each gas component was converted to Barrer ($10^{-10} \text{ cm}^3 \text{ (STP) cm cm}^{-2} \text{ s}^{-1} \text{ cmHg}^{-1}$). The theoretical selectivity was calculated as the ratio of gas permeabilities (p_i/p_j , where $p_i > p_j$), similar to our earlier article [\[19\]](#).

During binary gas experiments with CO_2/N_2 mixtures, feed and permeation chambers were initially flushed with N_2 and then closed. This step ensured that this particular gas had the same, 1 bar(a) pressure everywhere inside the cell. Thereafter, carbon dioxide was loaded to the feed compartment until a total pressure of around 1.7 bar(a) (0.7 bar(a) of CO_2 plus 1 bar(a) of N_2) was observed. At that point, because of the partial pressure difference of CO_2 between the sides (referred as the driving force), this molecule could begin the migration into the permeate chamber, while no transport of N_2 (background gas) had to be considered because of the equal nitrogen partial pressures on both membrane sides [\[27,28\]](#).

The CO₂ (commercial grade) and N₂ (>99.9 % purity) were products of Linde, Hungary. The permeation cell was thermostated at 37 °C.

3. Results and Discussion

3.1. Enzyme activity and its change in the presence of [Bmim][NTf₂] ionic liquid

The initial activity of the free CA enzyme was determined to be 3580 W-A unit mg⁻¹ protein by following the procedure introduced in Section 2.2. This, in the light of the data indicated by the manufacturer (3500 W-A unit mg⁻¹ protein), proved that the enzyme assays worked properly and the results obtained could be considered quite reliable, similarly to our previous study with biomass-derived CA enzyme preparation [15].

In case of the CA enzyme immobilized in the pectin-reinforced membrane, the initial activity measured was 9 W-A unit according to the modified procedure I in Section 2.2.. This, by taking into account the membrane surface corresponds to 1838 W-A unit m⁻², confirming that the CA was efficiently immobilized in the membrane.

So far, there has been an agreement in the literature studies that boosting the CO₂-separation in SILMs does not necessarily require great CA enzyme loadings. In recent investigations of Portuguese scientists, SILMs were successfully designed with as low as 0.1 mg CA/g IL enzyme

concentration [16,17], while Bednár et al. [15] demonstrated the appropriate performance of SILMs containing partly-purified CA enzyme preparation, obtained after plant biomass processing. Though longer-term experiments revealed the good time-stability of the enzymatically-accelerated membranes [15], no information regarding possible deterioration of CA activity in the presence of IL has been reported.

Following the modified procedure II in Section 2.2, we attempted to take a look into the enzyme-IL interactions. It turned out from the results that considerable loss of CA enzyme activity can be induced by the [Bmim][NTf₂] ionic liquid. Even as short contact time as 5 minutes caused an extreme, more than 90-95 % drop of relative enzyme activity. However², in accordance with measurements carried out after 10 and 15 minutes, stabilization of values could be noticed at around 0.5 % compared to the initial value.

From these observations, it would appear that depending on the properties of the ionic liquid, quick and notable inhibition/deactivation of the enzyme may take place and this phenomenon should be taken into consideration for process design. Supportive conclusions were made in our recent paper on the enzymatic hydrolysis of cellulose in the presence of [bmim][Cl] ionic liquid [24]. Nevertheless, even if only a smaller portion of the CA enzyme is preserved in an active form with time, it seems still be capable of doing the job that it needs to and facilitate CO₂-transport across the membrane. This might be attributed to the extremely high turnover number of CA (indicating the number of substrate molecules that is converted to product

through the catalytic site of particular enzyme within a given time period), which is reportedly around the magnitude of 10^6 s^{-1} , making it one of the most efficient enzymes in nature and a plausible candidate for biocatalytic CO_2 capture and sequestration [4]. This characteristic, at least for a certain degree, may compensate for the threat of rate-limitation in CO_2 -transfer when the number of active enzyme molecules decreases with time in the membrane. These results and considerations help to speculate why the performance of SILMs used in our previous work [15] demonstrated good time-stability (in terms of CO_2 and N_2 permeations) thorough a 4 week period. In brief, it can be supposed that the spinach-derived CA enzyme preparation initially underwent a remarkable activity loss due to the presence of $[\text{Bmim}][\text{NTf}_2]$, but despite, the residual number of working enzyme was still satisfactory to assure the enhanced CO_2 permeability and concomitantly higher CO_2/N_2 selectivity compared to the non-biocatalytic (control) membranes.

As the stability of the CA was concerned, in another set of experiments (where 1 mg CA enzyme – dissolved in 0.02 M Tris-HCl buffer, pH=8.3 – was entrapped in pectin beads) it was sought if the immobilization of enzyme in the pectin gel itself causes any notable drop of its beneficial properties (expressed as W-A unit/mL of pectin solution (2.5 wt%) in which CA was mixed and subsequently used for gelation in CaCl_2 (2.5 wt%) by allowing 12 h hardening time at slightly acidic pH). As a result, 13.1 W-A unit/mL pectin could be initially noted (according to modified procedure I in Section 2.2.) on the first day. Afterwards, although there was some loss of activity too with the time

elapsed, it was definitely much more less significant compared to that noticed in the presence of [Bmim][NTf₂]. In fact, after 3 weeks (during which beads were stored at 4 °C in 0.02 M Tris-HCl buffer, pH=8.3), the residual enzyme activity was still nearly 70-80 % of the initial. This experience that the majority of CA activity could be preserved for a longer time correlates well with our recent findings using free, biomass-derived CA enzyme preparation [15]. Accordingly, the application of pectin was not considered harmful for the CA enzyme.

3.2. Stability of pectin-containing membranes

Bubble-point porosimetry was applied to test stability of the membranes, in terms of their resistance against pressure. This technique enables the user to determine the pressure that exceeds the capillary attraction of a liquid in the biggest pore of a porous material [29]. During the measurements, the pressure of a gas (here N₂) is stepwise increased on the feed side of the membrane until a critical pressure (P_r) is reached, where the bubbles appear on the other side via the largest pore of the wetted material. This means in other words that the flux of the gas below P_r is negligible.

For the membranes reinforced with pectin in accordance with Section 2.3., the value of P_r was obtained as 7.2 bar. This, in comparison with the pectin-free control, presented a nearly 3-fold increase of pressure resistance. Therefore, it can be assumed that the pectin-supported membranes developed

in this work can be suitable for higher pressure gas separation task (>0.2 MPa transmembrane pressure difference), where conventional SLMs normally fail due to the instable membrane structure [22]. In our future investigation, such tests will thus be designed to evaluate CO₂-separation under such conditions.

In previous works of the literature, various SLMs were manufactured using ionic liquid and natural gelling agent i.e. gelatin [23]. It was found after taking stress-strain curves that membranes prepared only with gelatin (on porous cellulose support) reflected better mechanical properties (stress tolerance) than those containing both gelatin and IL (called Ion-Jelly[®] membranes). Moreover, gelatin-cellulose membranes could be characterized by an increased stiffness (based on the Young modulus) in comparison with the IL-containing ones [23].

3.3. Gas separation performance of pectin-reinforced membranes prepared with CA enzyme and lacking ionic liquid

As it was inferred in Section 3.1. that [Bmim][NTf₂] can cause the severe deterioration of CA enzyme activity, we aimed to study how the CA-boosted, pectin-supported membranes behave and perform in the absence of this IL during the permeation of pure as well as binary gases.

The results of gas permeation experiments are depicted in **Fig. 2**, according to which in case of the non-biocatalytic, pectin-containing cellulose acetate membranes the permeability of CO₂ was an order or magnitude higher

than that of N₂ (55 and 1.6 Barrer, respectively), which can be ascribed to their distinct solubility and diffusivity traits. Furthermore, it should be also noted that under pure/single gas conditions, no effect was taken on N₂ permeability by the presence of CA enzyme (1.6 vs. 1.7 Barrer). On the other hand, CO₂ permeability could increase significantly, from 55 to 93 Barrer. These outcomes match well with those trends communicated by [Neves et al. \[17\]](#), where it was found that both N₂ solubility and diffusivity (the two parameters that determine the permeability) remained unaffected by CA enzyme. Nevertheless, CA does able to positively influence CO₂ solubility coefficient [\[17\]](#), providing an explanation about the mechanism that could play a key-role in the improvement of the theoretical CO₂/N₂ selectivity (from 34 to 54 in the presence of CA enzyme).

It is also noteworthy that besides ionic liquid (in more general, solvent)-dependent enzyme inhibition/deactivation that may occur (Section 3.1.), the water activity in the membrane is also a factor that can affect the biocatalyst stability and efficiency [\[15-17\]](#). Hence, its variation (i) from system to system and (ii) with time is a possible reason leading to altered CO₂-separation performance. Thus, it means that an exact comparison of the already published literature might be done only for results obtained under standardized circumstances, in particular in terms of water activity (a_w). Although in this work a_w was not determined, we can suppose that it was quite high based on the report of [Basu et al. \[30\]](#), where it was deduced that in case of low methoxyl pectin (esterification degree < 50 %, which criteria is satisfied by the pectin

used in our work (29 %), as it can be seen in the Materials and methods) the equilibrium moisture content (g water/g dry matter) and water activity are interdependent. In fact, it was inferred by [Basu et al. \[30\]](#) that higher equilibrium moisture content will be accompanied by higher water activities in a wider range of temperature (30-70 °C). Since in the current paper the ratio between the mass of water and the mass of dry pectin was most likely above 0.3-0.5 at 30-40 °C (the interval where the temperature of gas separation tests falls), a_w in the pectin-reinforced membranes may have approached to the vicinity of 1.

Regarding the mixed gas tests conducted, it would appear that CO₂ permeability under these conditions was slightly enhanced from 93 to 102, using nitrogen as background gas. Though N₂ permeation between the cells was not considered (as described in Section 2.4), certain interactions between CO₂ and N₂ may have occurred inside the membrane related to nitrogen dissolved in the membrane material (cellulose acetate support as well as pectin matrix). However, we should also point to the fact that the approx. 10 % difference between pure- and mixed-gas CO₂ permeabilities may arise from experimental uncertainties, as it is more or less the confidence interval of the permeation measurements. Besides, it had been drawn by [Scovazzo et al. \[31\]](#) that mixed-gas selectivities in SILMs can be similar to those obtained with pure gases. These altogether suggest that further experimentation will be required (applying more gases i.e. H₂, CH₄ and their mixtures with CO₂) to unambiguously decide whether the observed differences of CO₂ permeability

under single- and binary-gas conditions are remarkable, and should stand in the scope of our next work on pectin-containing, biocatalytic membranes.

To demonstrate how the membrane performances fit to the recent trends, the pectin-reinforced gas separation membranes prepared with/without CA enzyme are illustrated against the Robeson upper-bound [32] in **Fig. 3**. As one can observe, this is a double logarithmic relationship, correlating how the CO_2/N_2 selectivity changes as a function of faster compounds (CO_2) permeability. We can see that the enrichment with CA was able to push the separation properties towards the upper-bound line, but further research is still needed for more attractive gas separation behavior of pectin-supported membranes.

So far, as it appears in **Fig. 1**, the permeation experiments were performed in rather short-terms (supposing that no significant dry out of the membranes occurred in the closed test cell). However, in longer terms, it is important to note that an issue may arise due to the evaporation of solvent (water) from the aqueous supported membrane when the membrane is coupled to a real gas separation process. In these cases, when the membranes are to be used to separate for example biologically produced gas mixtures (i.e. biohydrogen, biogas), it can be assumed that the humidity content of such gaseous streams (that are generated in a bioreactor via fermentation) would allow the prevention of this undesired phenomena. Therefore, in the continuation of this research, measurements will be dedicated to study this subject.

Conclusions

In this work, pectin-reinforced gas separation membranes containing carbonic anhydrase enzyme were prepared and studied. The results presented that the CA can lose majority of its initial activity in the presence of [Bmim][NTf₂] ionic liquid as a solvent candidate for supported membrane fabrication. Moreover, the pectin-containing membranes (lacking the ionic liquid possessing adverse effect on the biocatalyst) could be characterized with improved resistance towards higher transmembrane pressure conditions. The use of CA enzyme facilitated CO₂ permeation, and as a result, markedly enhanced CO₂/N₂ selectivity was achieved.

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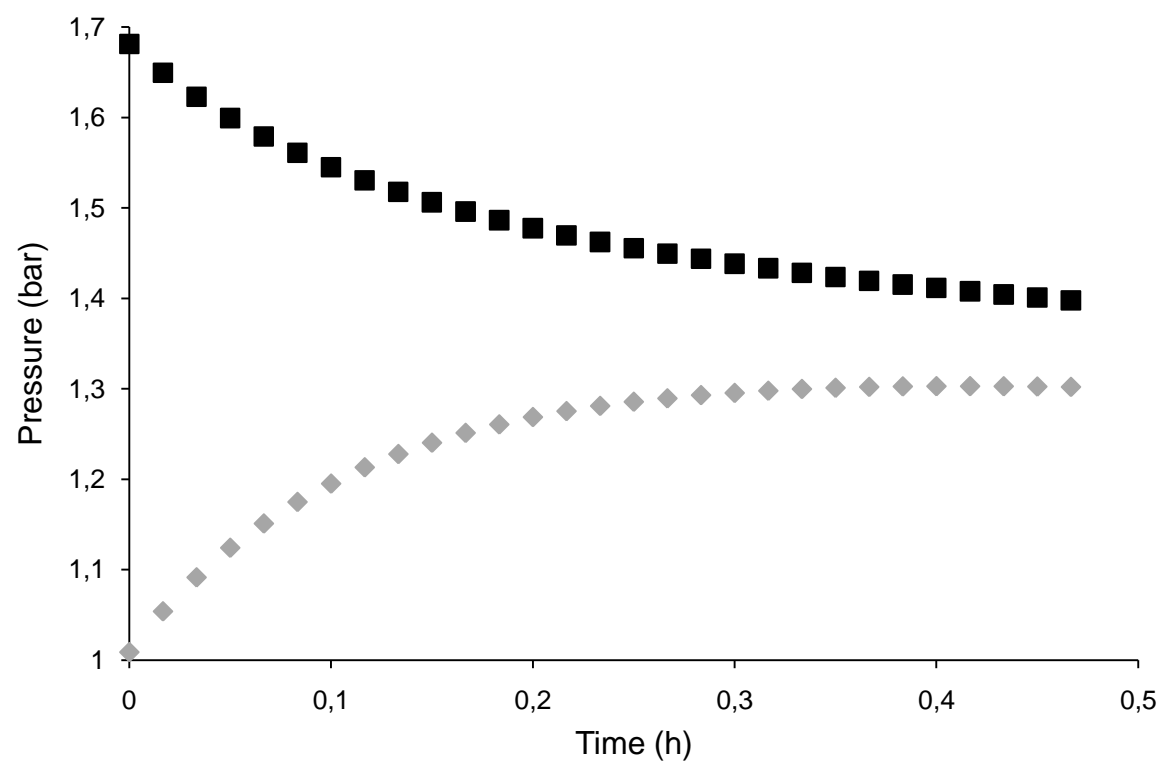
Figure legend

Fig. 1 – Progress curve of a typical gas permeation experiment. Square and diamond symbols represent the pressure in the feed and permeate cells, respectively.

Fig. 2 – Single/mixed gas permeabilities and CO₂/N₂ selectivity in pectin supported membranes with/without CA enzyme

Fig. 3 – The dependence of CO₂/N₂ selectivity on CO₂ permeability. Diamond and star symbols stand for the pectin-supported membranes with and without CA enzyme, respectively. The scattered line represents the Robeson upper-bound for polymeric membranes [32].

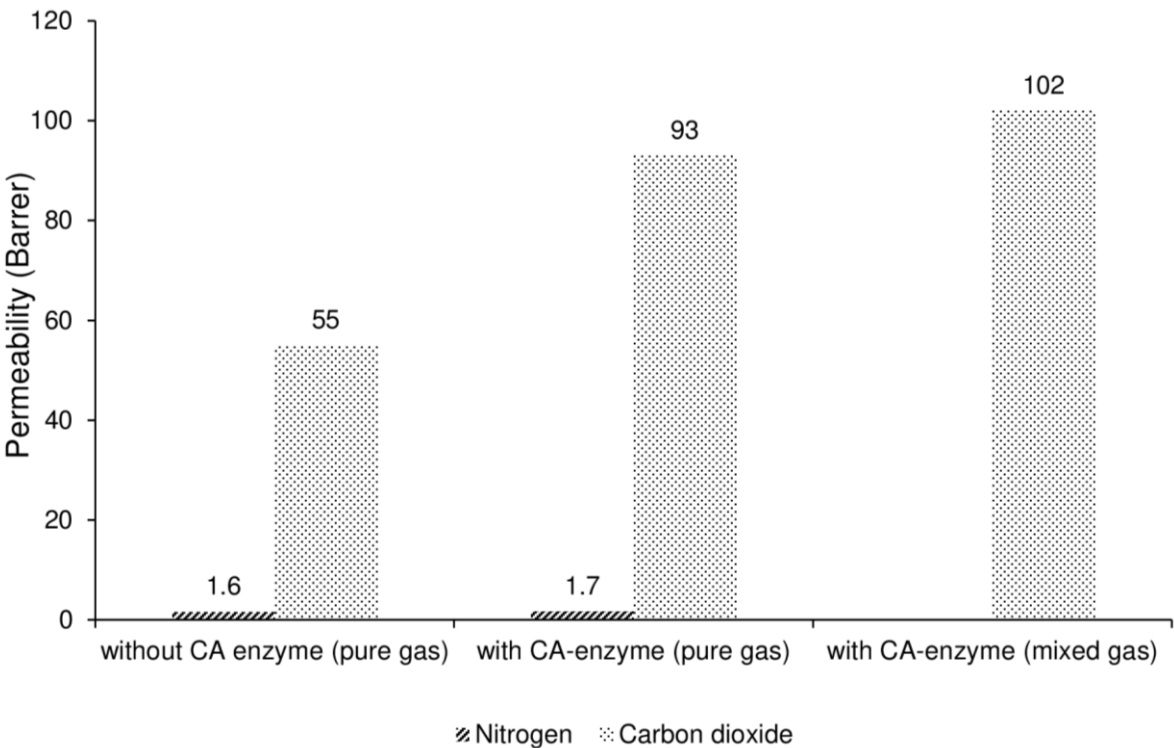
519 Fig. 1



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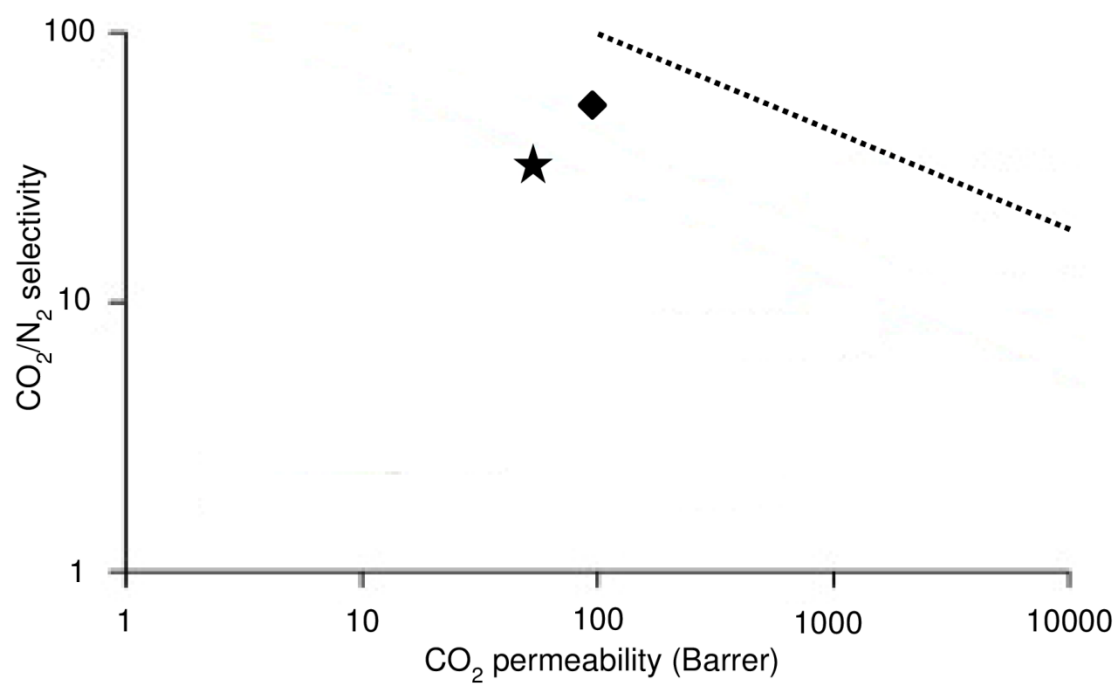
522 Fig. 2



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525 Fig. 3



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