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# Sammanfattning

Det växande behovet av hållbara produkter kräver att hållbara processer utformas och att hållbara råmaterial väljs redan från början. Det här arbetet syftar till att undersöka extraktionen av den biopolymer som är av intresse (BoI) från ett svampbaserat avfallsflöde. Ämnet är väl representerat i litteraturen, men en skalbar process är ännu inte väl etablerad inom industrin. Kombinationen av laboratoriearbete och processutveckling resulterade i en kompakt industriell processdesign bestående av alkalisk behandling, sur upplösning och fällning, vilket ger ett utbyte på 8,65 % av det teoretiskt tillgängliga innehållet. Behovet av en produkt med kontrollerad och fördefinierad viskositet kräver ytterligare tester samt analytiska metoder som säkerställer repeterbarhet och en heltäckande karakterisering av produktens egenskaper.

**Nyckelord**

Biobindemedel, Biopolymer, [redacted], [redacted], [redacted], Extraktion från fungi, Homogenisering, Industriella avfallsströmmar

# Abstract (IT)

La necessità crescente di prodotti sostenibili richiede la progettazione di processi sostenibili, e la scelta di materie prime sostenibili in primo luogo. Questo lavoro si pone l'obiettivo di investigare l'estrazione di un biopolimero d'interesse (BoI) da un sottoprodotto fungino di scarto. Questo argomento ha ampia disponibilità in letteratura, ma un processo applicabile su scala industriale non è ancora ben determinato. La combinazione di test di laboratorio e sviluppo di processo ha fornito un processo industriale compatto composto da trattamento alcalino, dissoluzione in acido e precipitazione che genera una resa del 8.65% del prodotto teoricamente disponibile.

La necessità di un prodotto con viscosità controllata e predeterminabile richiede test aggiuntivi, così come metodi analitici che assicurino ripetibilità e una caratterizzazione completa delle proprietà del prodotto.

**Parole chiave**

Biolegante, biopolimero, [redacted], [redacted], [redacted], estrazione da funghi, omogenizzazione, scarto industriale.

# Abstract (EN)

The growing need for sustainable products requires the design of sustainable processes and the choice of sustainable raw materials in the first place. The present work aims to investigate the extraction of the biopolymer of interest (BoI) from a fungal waste stream. This topic has wide literature available, but a scalable process is not well assessed in industry. The combination of laboratory work and process development provided a compact industrial design consisting of alkaline treatment, acidic dissolution and precipitation that yields 8.65% of the theoretical available content.

The need for a product with controlled and predefined viscosity requires additional testing, as well as analytical methods that ensure repeatability and a comprehensive screening of the product's properties.

**Keywords**

Biobinder, biopolymer, [redacted], [redacted], [redacted], fungal extraction, homogenisation, industrial waste streams.

# Confidentiality notice

This project contains confidential information that has been intentionally modified to meet OrganoClick's privacy and security requirements. To protect sensitive data certain words, paragraphs, and illustrations have been redacted, and specific technical terms have been replaced with broader terms like "biopolymer." These adjustments are made deliberately to ensure confidentiality, which may result in a reduction of detail. Readers should understand that the modified content is not an exact representation of the original material.

# List of abbreviations

| | |
|---|---|
| AcOH | Acetic acid |
| BM | Biomass ([redacted]) |
| BoI | Biopolymer of interest ([redacted]) |
| CM | Chemical Modification ([redacted]) |
| CP | Centrifugation Pellet |
| DC | Dry Content |
| DW | Dry Weight |
| DCM | Degree of Chemical Modification ([redacted]) |
| DI-H2O | Deionized water |
| EtOH | Ethanol |
| EXP | Experiment |
| HCl | Hydrochloric acid |
| LaOH | L - (+) - Lactic acid |
| M1 | Monomer 1 ([redacted]) |
| M2 | Monomer 2 ([redacted]) |
| MW | Molecular Weight |
| OC | OrganoClick AB |

| | |
|---|---|
| PB | Precursor Biopolymer of BoI ([redacted]) |
| RT | Room Temperature |
| SDG | United Nations’ Sustainable Development Goals |
| SN | Supernatant |
| SB | Secondary biopolymer ([redacted]) |

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# Sommario

## S1. Introduzione

Il presente lavoro descrive lo studio sperimentale e di letteratura per l'estrazione di un biopolimero di interesse (BoI) da un prodotto di scarto dell'industria alimentare. Il progetto si pone nel contesto più ampio dell'azienda OrganoClick AB (OC) con sede in Täby (Contea di Stoccolma, Svezia) dove, in linea con gli obbiettivi dello sviluppo sostenibile delle nazioni unite SDG9 e SDG14, si commercializzano sostituti organici di prodotti normalmente a base plastica.

Il BoI è un componente del complesso polielettrolitico presente in OC-BioBinder®, un sostituto organico ai tradizionali leganti plastici usati nel tessuto non tessuto. L'obbiettivo è quello di progettare un processo di estrazione affidabile e sostenibile per avviare la produzione in loco del biopolimero, con lo scopo di ridurre le spese correlate al suo approvvigionamento.

## S2. Contesto

Il tessuto non tessuto è un materiale costituito da fibre assemblate in una tela continua, e le cui proprietà sono determinate dall'uso dei leganti chimici, comunemente costituiti da etilene vinil acetato. Il BoI, utilizzato come alternativa organica all'interno degli OC-BioBinder, è presente nella parete cellulare di molti funghi (micro e macroscopici) nella forma di un biopolimero precursore (PB). Il PB è poi convertito in BoI per mezzo della reazione con soda caustica concentrata.

La parete cellulare è principalmente costituita da carboidrati (circa il 70%), affiancati da un 20% di proteine e altri costituenti minori, tra cui lipidi, minerali e acidi organici. Tra i carboidrati, tuttavia, solo un approssimativo 15% è assimilabile a PB, mentre almeno il 48% è costituito da un biopolimero secondario (SB), a cui PB è legato covalentemente (Krake et al., 2024).

Proprietà importanti del BoI sono la sua solubilità in molte soluzioni acide che acquisiscono una crescente viscosità dipendente dalla concentrazione di BoI, e da altre proprietà del polimero come la sua massa molecolare e dalla sua composizione monomerica.

Un parametro che determina le qualità del BoI è il grado di modificazione chimica (DCM) che può variare da 0 (l'intera catena polimerica è composta da soli monomeri di tipo 1 (M1)) a 100 (solo M2). Valori bassi del DCM corrispondono ad un polimero di tipo PB, mentre un polimero assimilabile al BoI e solubile in condizioni acide è corrispondente a valori di DCM minimi di circa 50-65 ([redacted]; Komi, 2017).

Il processo di estrazione (schematizzato in Figure 6) sfrutta principalmente tre step, tra cui un trattamento alcalino, volto sia alla separazione del PB dal SB, che alla conversione di M1 in M2 o analogamente da PB a BoI. Il supernatante (SN) viene poi separato dal pellet di centrifugazione (CP) che è disciolto in soluzione acida per sfruttare la proprietà di solubilità specifica del BoI. Infine, il SN acido è poi precipitato mediante l'aggiunta di NaOH fino al raggiungimento di un pH basico che permette di estrarre auspicabilmente puro BoI nel CP finale che può essere infine lavato fino a neutralità.

Tra i metodi analitici utilizzati, vi è la spettroscopia infrarossa a trasformata di Fourier con riflessione totale attenuata (ATR-FTIR) che sfrutta gli specifici picchi di assorbimento/trasmissione che si possono associare allo stiramento e flessione di diversi gruppi funzionali. Questa informazione è poi utilizzata per stimare la purezza del campione, associata ad esempio alla presenza di impurezze proteiche o lipidiche.

È inoltre sfruttato un test per determinare la presenza di M2. Il metodo prevede un'idrolisi di 24 ore in HCl in concentrazione di 2 mol/l, che rilascia monomeri M2 siano essi originariamente presenti in forma polimerica, monomerica, e covalentemente legata a SB. Inoltre, i monomeri M1 durante lo step in HCl sono anch'essi convertiti in M2. Gli step successivi prevedono l'addizione di acetilacetone e reagente di Ehlrich (DMAB), che permettono di sviluppare un colore rosso che è quantificato nello spettrofotometro UV-vis per mezzo della sua assorbanza a 530 nm (luce verde).

Infine, la viscosità di una soluzione acida del polimero prodotto è determinata con un viscosimetro rotazionale.

## S3. Metodo

Il processo di estrazione, come anticipato, si basa su tre step principali (trattamento alcalino, acido e precipitazione alcalina) intervallati da centrifugazione per separare la frazione solubile nel SN da quella insolubile nel

CP. Diverse combinazioni di parametri di concentrazione, temperatura e tempo di trattamento sono utilizzati negli otto principali esperimenti. Il trattamento alcalino è stato preceduto da una prima omogenizzazione in acqua dal secondo esperimento in poi. Dal momento di addizione di NaOH per una concentrazione variabile tra 1 mol/l a 50% m/m (19 mol/l) si è continuata la miscelazione con impeller e omogenizzazione per un tempo compreso tra 50 minuti e 2 h. Eccezion fatta per l'EXP7 dove la prima omogenizzazione in acqua è effettuata con una concentrazione di 0.5 mol/l e il trattamento ad alta concentrazione di alcali è più breve (10 min). La temperatura è controllata per mezzo di una piastra riscaldante munita di termostato. Gli step di lavaggio del CP1 sono introdotti dall'EXP3.2 effettuati con successive addizioni di acqua e centrifugazione fino alla neutralizzazione dell'acqua di lavaggio (pH misurato con cartine al tornasole).

L'estrazione in acido è stata performata a 60°C o temperatura ambiente principalmente in acido acetico (AcOH), ma anche acido lattico (LaOH) e HCl sono stati testati. La precipitazione del prodotto è ottenuta aggiungendo NaOH in soluzione acquosa fino al raggiungimento di un pH basico.

La centrifugazione è effettuata in due centrifughe di scala diversa (2.6 l e 300 ml) a seconda della scala dell'esperimento alla massima velocità di rotazione (rispettivamente 4600 e 4000 rpm) per il tempo necessario a creare un pellet compatto.

I parametri specifici per ogni esperimento sono schematizzati in Table 1.

## S4. Risultati e discussione

Tra i principali risultati ottenuti da un test di solubilità effettuato su BoI commerciale vi è l'ampia compatibilità con gli acidi organici (AcOH e LaOH), che presentano solubilità di almeno 20 mg/ml per concentrazione di acido tra l'1% e rispettivamente il 60% e l'80% (massime concentrazioni dei due acidi disponibili in azienda), mentre la solubilità in acido cloridrico è limitata nel solo range tra lo 0.3% e il l'1%. Questo risultato, unito alla maggiore quantità di PB (determinato essere PB e non BoI dal suo spettro FTIR) estratta con LaOH in EXP3.4 rispetto a 3.3 (con AcOH), permettono di preferire AcOH nell'estrazione acida.

L'efficacia dell'estrazione è valutata principalmente per mezzo della quantificazione delle perdite di monomero in SN1 (prodotto di scarto del trattamento alcalino) e CP2 (frazione non dissolta in acido) con il saggio DMAB, affiancata dall'osservazione dello spettro FTIR del prodotto finito. La valutazione delle quantità presenti nelle suddette perdite è tenuta in considerazione come segue. Grandi quantità di M2 rilevate in SN1 ed eventualmente nelle acque di lavaggio (si ricorda che la molecola rilevata è M2, ma essere può essere proveniente nel campione esaminato in forma di M1, M2, PB o BoI, in quanto il primo step dell'analisi consiste in idrolisi in acido che depolimerizza PB e BoI, e modifica chimicamente M1 in M2) indicano che le condizioni nello step alcalino sono troppo severe, causando depolimerizzazione del prodotto a tal punto che esso diventa solubile in acqua e soluzioni basiche.

Le perdite in CP2, d'altro canto, possono essere correlate all'inefficienza della dissoluzione acida dove BoI non è sufficientemente solubile (pH, tipo e concentrazione di acido, temperatura), oppure al fatto che la soluzione è satura di BoI causandone parziale precipitazione. Entrambe queste problematiche sono intrinsecamente risolte utilizzando una soluzione acida in cui il biopolimero commerciale è solubile, ed un rapporto solidi:soluzione sufficientemente basso. Un'altra spiegazione per le perdite di M2 in CP2 è che le condizioni nello step alcalino siano troppo blande, con la conseguenza che il polimero non è sufficientemente modificato chimicamente oppure ancora legato nella struttura con il polimero secondario. Entrambi questi casi presentano un prodotto non solubile in acido e richiedono maggiore tempo, temperatura o [NaOH] nel primo step.

La sola estrazione di un polimero scarsamente solubile e dallo spettro simile a PB negli EXP da 1 a 4, fanno constatare la scarsa presenza di BoI nella biomassa. Questa la ragione per cui nei test successivi viene effettuato il trattamento alcalino in concentrazioni più alte, rispettivamente del 50 e 40% come utilizzato in letteratura per la reazione di modificazione chimica. Questa modifica riduce la frazione persa in CP2 dall'ordine del 70% a circa il 30%, con l'ottenimento di un pellet finale solubile e dallo spettro FTIR con picchi assimilabili a quelli del BoI commerciale. La maggiore perdita di prodotto in SN1 e acque di lavaggio è una naturale conseguenza delle condizioni più severe nello step alcalino. Trovare parametri ottimali per massimizzare la quantità di prodotto e minimizzare le perdite in soluzione alcalina è chiaramente punto centrale del problema, ma richiede tutt'ora che una maggiore quantità di test venga effettuata.

Gli step di lavaggio di CP1, introdotti da EXP3.2, sono risultati essenziali per il processo, in quanto il biopolimero non risulta solubile quando la soluzione è molto ricca di sali (acetato di sodio) per via di un fenomeno di salting-out. Inoltre, l'introduzione di questo step permette di risparmiare una importante quantità di acido, siccome in assenza dei lavaggi, l'alta concentrazione di NaOH richiede un altrettanto grande volume di soluzione di acido debole per la neutralizzazione e successiva acidificazione.

Una descrizione dettagliata dell'interpretazione degli spettri FTIR è disponibile nella sezione 4.7, mentre in questo paragrafo viene solo fatto presente che gli spettri dei prodotti degli esperimenti da 3.3 a 8 sono confrontati a quelli di BoI e PB commerciali. Il risultato è, come anticipato, uno spettro analogo a quello del

PB in EXP3, EXP4 e EXP7, lasciando intuire la necessità di maggiore CM in queste condizioni. In letteratura, è inoltre ampiamente descritto l'uso dello spettro FTIR per la stima quantitativa del DCM a partire dal rapporto di picchi caratteristici dei biopolimeri. Questa strada è stata percorsa applicando le equazioni a diversi BoI commerciali con noti DCM. L'analisi, i cui dettagli sono riportati in Appendix 1, è risultata in una scarsa affidabilità di questa tecnica, con l'ottenimento di un $R^2$ massimo di 0.57.

Una soluzione del prodotto dell'EXP8, corrispondente all'unione di tre batch con parametri pari a quelli di EXP6 è poi testata per la sua viscosità. Il risultato è un prodotto classificato a bassa viscosità, ma che può essere utilizzato in alcune formulazioni di OC-BioBinder®.

La produzione di una di queste formulazioni, inoltre, è risultata in un prodotto finito dalle caratteristiche allineate a quelle dello stesso prodotto a partire da BoI commerciale nello stesso range di viscosità.

## S5. Design del processo industriale

Un importante aspetto del progetto è quello di disegnare il processo anche su scala industriale. Il design è basato sui parametri degli EXP6 e EXP8, in quanto i loro prodotti hanno caratteristiche più simili al BoI commerciale in termini di purezza (determinata con il metodo DMAB), spettro FTIR e solubilità.

Il progetto è effettuato su una scala di 250 kg di biomassa, che con una resa sulla biomassa prevista pari all'1% (come ottenuta nei sopraelencati esperimenti) porterebbe alla produzione di 2.5 kg di BoI con un tempo batch totale di 21 ore. La produzione inizia in un serbatoio adiabatico da 5 $m^3$ di acciaio resistente alla corrosione, equipaggiato di omogeneizzatore, miscelatore e condensatore per i vapori. Qui avviene in primo luogo l'omogeneizzazione in acqua per 3 ore, seguita dall'aggiunta di soda fino alla concentrazione finale di 11.5 mol/l con trattamento alcalino della durata di 50 minuti. La presenza del condensatore per i vapori permette il mantenimento controllato della temperatura del sistema al suo valore di ebollizione (misurato approssimativamente pari a 110°C).

La soluzione è poi inviata ad un serbatoio di 15 $m^3$ di HDPE munito di miscelatore e già ricaricato di 12.75 $m^3$ di acqua di lavaggio. Il sistema viene continuamente inviato in centrifuga. Qui il supernatante è inviato al sistema di trattamento delle acque, mentre il pellet ricircolato al serbatoio di lavaggio. Il ciclo di lavaggio si conclude quando il pH dell'acqua di scarto (misurato con un sensore di pH in comunicazione con una valvola a tre vie) raggiunge la neutralità. Il pellet è quindi inviato al serbatoio per la dissoluzione acida insieme alla dose di AcOH per una concentrazione finale di acido pari al 2%. Questo processo, della durata di tre ore è seguito dalla rimozione della fase insolubile via filtrazione, e successiva essiccazione spray del supernatante contenente il BoI.

I consumi complessivi di questo processo comprendono, per 282.5 kg di biomassa (con contenuto secco pari all'88.5%), 1287.9 kg di NaOH in pellet, 47 tonn di acqua e 47.2 kg di AcOH 60%.

L'acqua di lavaggio è inviata al sistema di recupero presente nell'impianto, ed è ricircolata nel sistema. Può essere ulteriormente investigato un metodo per il recupero della soda, per esempio tramite la precedente flocculazione dell'acqua di lavaggio per rimuovere i residui cellulari in modo da ottenere una polvere principalmente costituita da NaOH all'evaporatore. Il pellet insolubile in acido è trattato esternamente per la produzione di biogas, così come il potenziale solido prodotto dalla flocculazione. L'uso dell'essiccazione spray può permettere anche il ricircolo dell'AcOH se i vapori del ciclone sono sottoposti a condensazione.

## S6. Conclusioni

In conclusione, il lavoro descrive la combinazione dello studio di letteratura, test di laboratorio e design di processo per l'estrazione di BoI da biomassa fungina, risultante in una resa dell'1% sulla materia prima trattata. Il processo consiste in uno step di trattamento alcalino, un'estrazione acida e la finale separazione del prodotto finito dal supernatante via precipitazione o essiccazione spray. Le perdite principali sono rilevate nel supernatante del trattamento alcalino, indicando che le condizioni molto rigide causano parziale depolimerizzazione del materiale. D'altro canto, una quantità rilevante di prodotto è presente anche nel pellet di dissoluzione acida, frazione che può essere ridotta aumentando la concentrazione o il tempo di contatto con NaOH.

Il prodotto del processo con le condizioni attuali è di categoria a bassa viscosità e può essere utilizzato con successo negli OC-BioBinder®, tuttavia una quantità maggiore di test è necessaria se si vuole sviluppare un processo più efficiente e dalle qualità del prodotto finito predeterminabili.

Allo stesso tempo, maggiori studi sono necessari in termini analitici. L'attuale uso del saggio sul monomero prevede l'utilizzo di un range di calibrazione invece della più intuitiva e classica retta di calibrazione per via dell'incompleta idrolisi del polimero nel primo step del saggio. Ottimizzare questo step in modo da ottenere rette coincidenti a partire da M2 e PB commerciali permetterebbe una maggiore accuratezza dei risultati. Maggiore quantità di test è necessaria anche in termini di determinazione del DCM, che attualmente non può essere effettuata in azienda.

Un altro punto interessante del progetto è l'investigazione dell'estrazione da diverse biomasse. In questo lavoro si è studiata l'estrazione da una sola biomassa fungina, mentre dagli studi di letteratura si riconosce la presenza di biopolimero in varie altre biomasse fungine. Andare a fondo di questo aspetto potrebbe essere utile sia in termini qualitativi, permettendo la potenziale estrazione di polimero a viscosità diversa, che economici nel caso essi permettano minori consumi o di lavorare a concentrazioni più elevate di biomassa, e conseguente minore volume a pari quantità di prodotto. La varietà di materia prima utilizzabile aumenta anche la robustezza del processo in quanto consente di mantenere la produzione anche con ipotetiche variazioni del mercato della biomassa.

# 1. Introduction

It is well known that the implementation of the United Nations' Sustainable Development Goals (SDG) is vital to ensure harmony and prosperity for people and the environment. With particular emphasis on SDG9 (industry, innovation, and infrastructure), SDG12 (responsible consumption and production) and SDG14 (life below water), growing interest is developing towards sustainable alternatives to plastics and fossil-based materials (United Nations, 2015).

To this end, OrganoClick AB (OC) commercializes biobased substitutes for plastic binders that are commonly present in chemically bonded nonwovens. OC-BioBinders® contain a polyelectrolyte complex of different biopolymers, of which one in this report is referred to as the biopolymer of interest (BoI). [redacted].

At OC, the BoI is currently supplied by a waste stream of the food industry, allowing the production of high value-added products reducing the pollution caused by their disposal.

However, since the current source is seasonal dependent and the BoI is produced on the other side of the world, which makes it expensive due to long transportation, the present work aims to investigate and design an extraction process for the BoI from an alternative waste stream, with the purpose of assessing the sustainable and cost-effective production in-house of the binders' key component.

# 2. Background

## 2.1. OrganoClick AB

OrganoClick AB is a Swedish green chemistry company that focuses on replacing hidden plastics and toxic chemicals in materials such as paper, wood, textile, and nonwoven.

Nonwovens are engineered materials where fibres (either synthetic or 100% pulp) are bonded together to form a homogeneous and continuous flat web. Used in many different applications like napkins, diapers, tea and coffee bags, nonwovens require specific mechanical and physical properties such as wet and dry strength and water and oil resistance. These qualities are traditionally given to the material by impregnating it with fossil-based polymeric binders that usually contain ethylene-vinyl acetate and may contain PFAS.

At OC, a new patented biodegradable binder is produced starting from high-volume waste streams of the food industry, enabling natural materials, which would be incinerated or sold as feed, to be repurposed into new value-added materials.

## 2.2. The biomass

It is found that the BoI is present in the cell wall of many fungi and mushrooms mostly in the form of a precursor biopolymer (PB) ranging from 1 to 40% of their dry weight (Su et al., 1997). This precursor form can be converted to the BoI with an alkaline treatment in NaOH, as it is done industrially also in the predominant production of BoI.

The biomass that is investigated in this work, that after this introduction is going to be simply referred to as biomass (BM), was chosen because it is technologically well characterized, and it is the main component (96-98%) of a cheap and highly available side stream of a large-volume industry.

The structure and shape of the fungal cell is given by its wall, which provides physical strength and protection from osmotic pressure (Gooday, 1984). Its polysaccharidic structure accounts for 20-40% of the cell's dry weight (Lamon et al., 2023). The overall composition of the target BM is strongly dependent on the mycelial strain and age and its culture medium ([redacted], [redacted]) but can be approximately summarized as in Figure 1 (Krake et al., 2024). The main component of BM is a secondary biopolymer (SB), present as 48.6 ± 1.4% of the cell's dry weight. Other constituents are proteins (22.2 ± 0.7 %), other carbohydrates and glucose containing materials (9.4 ± 1.9%), fats (1.4%), organic acids (1%) and other smaller fractions such as minerals. In general, the PB is likely present in the BM in a fraction between 11 and 20% ([redacted] [redacted]; Brauer et al., 2023).

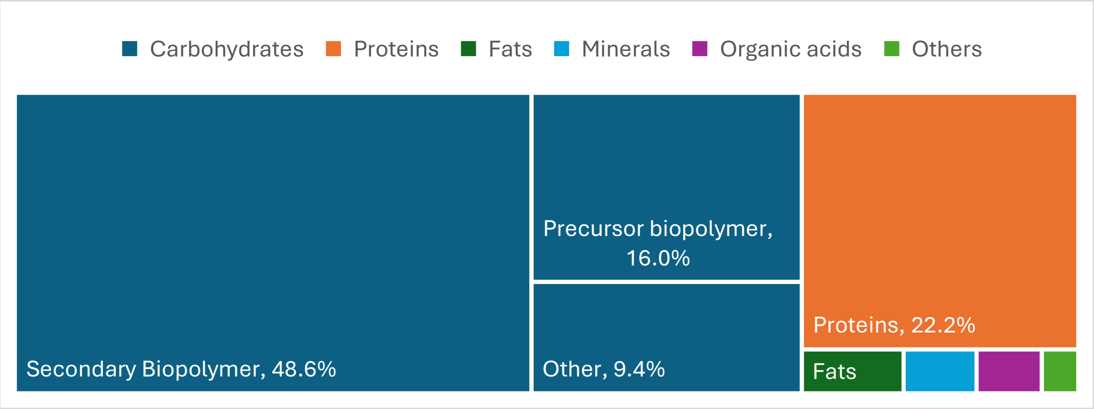

*Figure 1: BM composition (Krake, 2024)*

Moreover, the extraction can yield up to 11% of the DW even if some realistic values are closer to 4 - 8% (Dhillon et al., 2013; Abdel et al., 2017).

## 2.3. Biopolymer of interest

Known for its biocompatibility, biodegradability, and antimicrobial properties (Sarfraz et al., 2024), BoI has the peculiar characteristic of being soluble in acidic conditions, (pKa ≈ 6.44 according to Giraldo et al., 2021) allowing it to be dissolved in aqueous liquids as long as the pH is kept in the acidic range.

In fungal biomasses, PB is found in the structure of the cell wall, covalently bonded to the SB. More specifically, they form an alkali-insoluble complex that is not readily separated in mild aqueous solution.

The BoI is industrially derived from PB, [redacted] [redacted]. The conversion is done with highly concentrated NaOH, where the monomers prevailing in PB (M1) are progressively converted to M2, characterized by a molecular weight (MW) that is approximately 20% lower than M1.

$$MW_{M2} = 0.8 * MW_{M1} \quad (1)$$

The polymer is defined as BoI when it gets soluble in aqueous acidic media. This usually happens when in the polymer backbone more than about 50-65% of the monomers are M2. Similarly, when the polymer is insoluble with most of its monomers being M1, the polymer is defined PB ([redacted]; Komi, 2017). The percentage of monomers in the polymer chain that are M2, is called degree of chemical modification (DCM) and this value can range from zero (every monomer is M1) to one hundred (every monomer is M2).

In presence of an acid like acetic acid (AcOH), a basic side group on BoI gets protonated, causing the polymeric chains to be positively charged and electrostatically repel each other. This phenomenon leads to chain separation, and thus solubilization when the cohesion forces are overcome by the coulombic repulsion between cationic side groups (Giraldo et al., 2021).

PB is generally not soluble in aqueous media, however its solubility not only depends on the DCM, but also on the disposition of the monomers and its molecular weight. The polymer generally gets soluble increasing DCM and lowering its MW (Giraldo et al., 2021). [redacted], affirms that BoI has a pKa of approximately 6.5 independently of its DCM in the range 75 - 100.

In general, fungal BoI naturally presents a lower molecular weight than that of traditional sources. According to Dhillon et al. (2013), BoI from traditional sources exhibits a molecular weight approximately $10^2$ times greater than the average fungal counterpart. The molecular weight of BoI is important due to its direct influence on the viscosity of the solution in which it is dissolved, which in turn determines its physical and chemical properties. Therefore, measuring the viscosity of the product is a vital step in the process, as it helps determine the suitable formulation for its application.

## 2.4. Project background

The present project is the prosecution of the work previously done by Hul (2024) and Baynham et al. (2024) in their Master's and bachelor's thesis projects respectively. In these works, each step in the extraction process was focused on the removal of specific components in the mixture, in order to get pure BoI as the final product.

In particular, the tests performed by Hul, 2024 are presented in Figure 2. They included cell lysis in distilled water (DI-H2O) via ultrasonication at high T to denature proteins, followed by PBS washing to remove nucleic acids, cell wall proteins and the soluble fraction of the SB. An ethanol (EtOH) washing step to remove lipids and proteins, water washing to remove residuals of PBS and EtOH. Next, the CM step in NaOH solution to convert from PB to BoI followed by washing with water, neutralization and drying.

Moreover, an enzymatic degradation and acidic dissolution steps were investigated by Baynham et al. (2024) aiming respectively to further release PB from the cell wall and to remove some impurities such as PB residuals and SB, exploiting the solubility in acid of BoI.

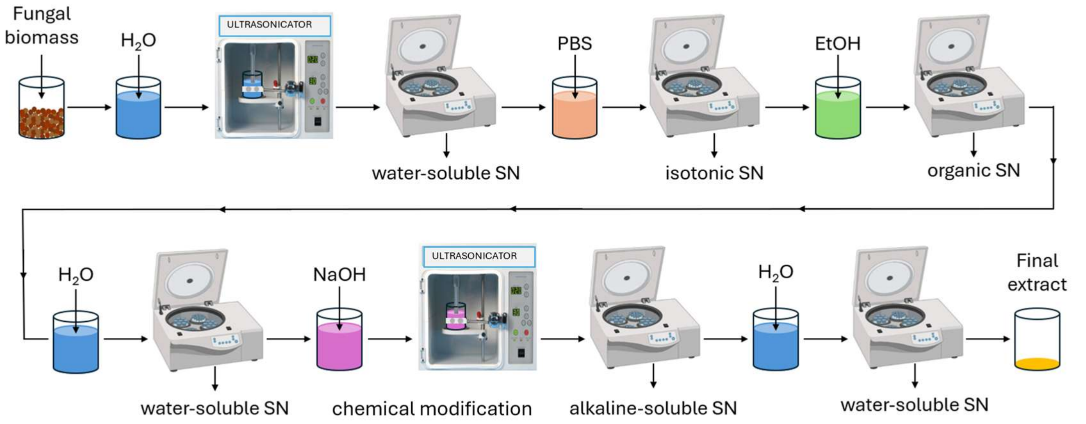

*Figure 2: Process as ideated and performed by Hul (Hul, 2023).*

It has been observed that extracting BoI from fungal biomasses mainly needs three steps: hot alkaline treatment, acidic dissolution on the alkali insoluble fraction and precipitation of the acidic soluble fraction (Rane et al., 1993; Abdel, 2017). This allows lysis and CM in the first alkaline step, and purification of BoI from SB in the acidic dissolution. It is on that principle that the process was designed and performed in the present work. A flow chart of this kind of process is shown in Figure 3.

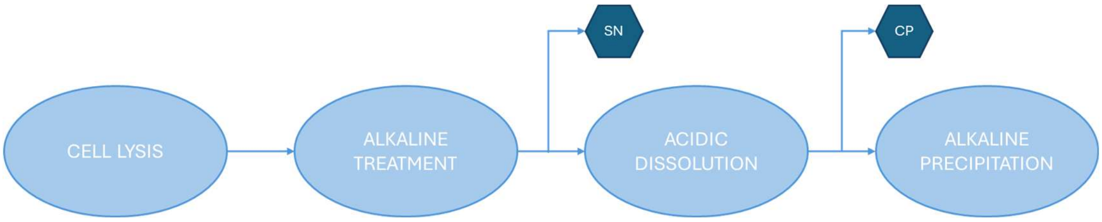

*Figure 3: Simplified process scheme*

Ideally, the alkaline step allows on one side to denature proteins and nucleic acids, leaving the system in the first supernatant, while also breaking the covalent bonds between PB and SB, and chemically modifying the PB to BoI. Therefore, this simplified process would leave unbonded BoI in the first centrifugation pellet (CP), which is then purified from SB and PB residuals in the acidic dissolution step, exploiting the special acid solubility property that BoI does not share with PB and SB. Finally, the pH of the acidic supernatant (SN) is shifted to basic with addition of an NaOH solution, causing precipitation of the target biopolymer. The separation of the solutions from insoluble fractions is done through centrifugation.

This kind of process shows good scalability perspective thanks to its compact scheme, and it is for this reason, together with the wide literature availability, that this process setup is investigated in the present work, with a particular focus on optimization of the lysis-alkaline treatment and acidic dissolution steps.

## 2.5. Analytical methods

Many analytical methods are used to assess the effectiveness of each step in the process. In particular, the characterization of the final pellet and the discarded fractions (alkaline SN and acidic centrifugation pellet (CP)) was done in house with the methods that are listed here.

### *2.5.1. FTIR*

The Fourier Transform Infrared spectrometer with attenuated total reflection (ATR-FTIR) is used to estimate the composition of the samples, with a particular focus on the identification of impurities such as proteins and unwanted carbohydrates.

In the FTIR spectrometer IR radiations in the range 400-4000 $cm^{-1}$ hit the samples, causing specific adsorption and/or transmission of energy in the vibration of the bonds. Diverse kinds of vibrations happen within a bond, such as the symmetric stretch, antisymmetric stretch, bending, rocking, wagging, and twisting. Since these vibrations match those of IR light, this method allows to identify certain groups based on the peaks (or valleys) in the FTIR spectrum. The spectrum can be divided into four main regions (Nandiyanto et al., 2019):

- Single bond region: 2500-4000 $cm^{-1}$ (O-H, N-H, C-H)
- Triple bond region: 2000-2500 $cm^{-1}$ (C≡C, C≡N)
- Double bond region: 1500-2000 $cm^{-1}$ (C=C, C=O, C=N)
- Fingerprint region: 600-1500 $cm^{-1}$

In Figure 4 some relevant functional groups and their specific adsorption bands are schematized, where the darkness of the grey shade corresponds to the intensity of the absorption peak (InstaNANO, 2025; Jasco, 2025).

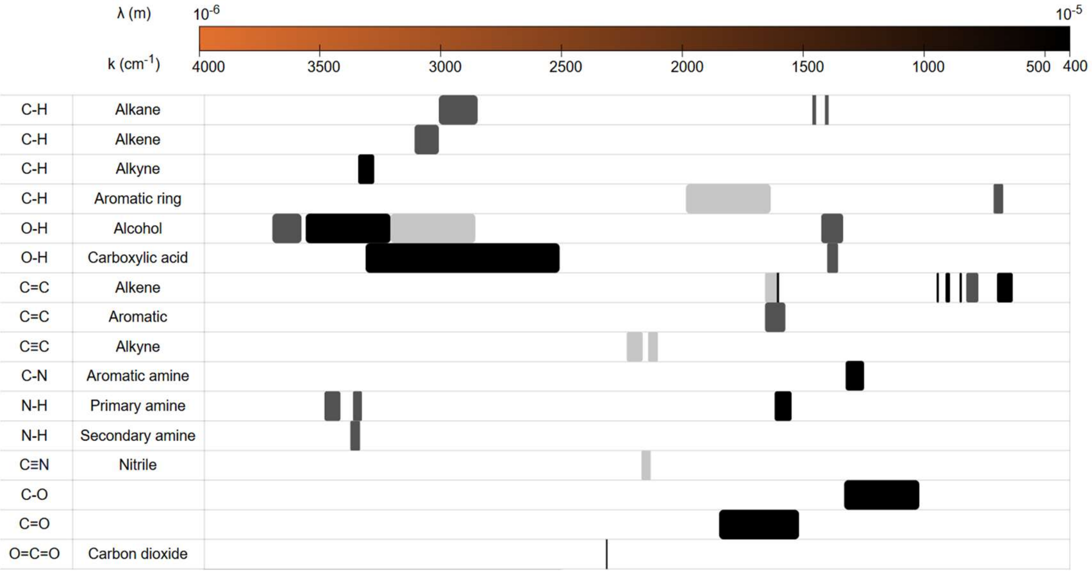

*Figure 4: Functional groups and corresponding absorption bands (InstaNANO, 2025; Jasco, 2025).*

### 2.5.2. Monomer assay by UV/VIS

A colorimetric method for the detection of M2 was also assessed and implemented.

This assay is based on the reaction of the monomer M2 with acetylacetone and p-Dimethylaminobenzaldehyde (DMAB, aka Ehrlich's reagent), developing a red colour that is quantified in the UV-Vis spectrophotometer by its absorbance of the green light. In particular, M2, reacting with acetylacetone forms a pyrrole derivative, that in contact with DMAB originates a stable colour, whose absorbance at 530 nm is proportional to the concentration of M2 in the sample ([redacted]; Belcher et al., 1954).

The method consists of hydrolysis of the sample in 2 mol/l HCl, followed by neutralization, dilution with water, addition of acetylacetone reagent and DMAB reagent, incubation with ethanol and measurement of absorbance.

In the hydrolysis step, both PB and BoI get lysed to their monomers, and M1 is chemically modified to M2. Consequently, the method detects M2 indifferently coming from monomers, PB or BoI. It is given to the interpretation of the experimenter, and the cross validation with other analytical methods (ex. FTIR), the assessment of which polymeric/monomeric material is expected to be present in the sample.

The absorbance values are then compared with a calibration curve made with commercial M2, PB and/or BoI. Figure 5 shows the Eppendorf's tubes with the samples of commercial BoI for the calibration curve, and the ones for EXP4's assay.

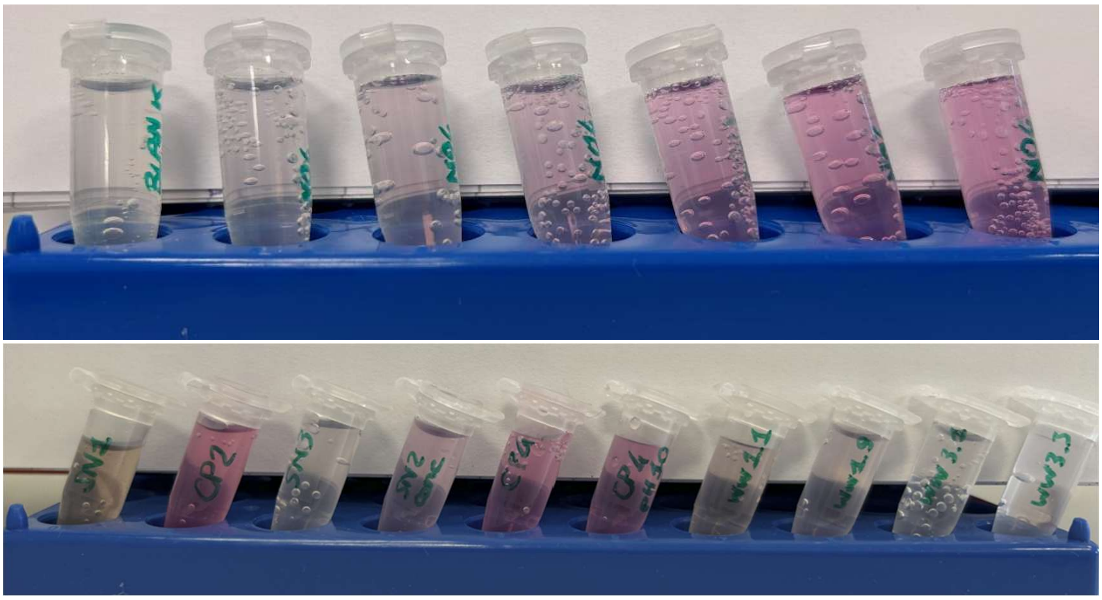

*Figure 5: (a) Calibration curve of commercial BoI and (b) samples from EXP4.*

### 2.5.3. Viscosity

Given the importance of viscosity in determining the functional properties of BoI, the dry final pellet is dissolved in an acidic solution and analysed using a rotational viscometer. The measured viscosity value is then used to determine the formulation in which the product can be used. The viscosity is strongly related to the molecular weight (MW) of the product and to its DCM. Therefore, key step of the process is to focus on controlling the MW while improving purity and CM.

The MW can be estimated from the viscosity with the Mark-Houwink equation Eq. (5), where $[\eta]$ expressed in $ml/g$ is the intrinsic viscosity, and $M_v$ the viscosity average molecular weight.

$$[\eta] = K * M_v^{\delta} \tag{2}$$

The constants K and δ are specific of each polymer. For BoI they are a function of the DCM as reported by Wang et al. (1991, from Qian et al., 2023):

$$K = 1.64 * 10^{-3} \quad * DCM^{14} \tag{3}$$

$$\delta = -1.02 * 10^{-2} * DCM + 1.82 \tag{4}$$

Between the equations for determination of intrinsic viscosity from a single point (Kwaambwa H.M., 2006):

$$[\eta] = \frac{1}{c}\sqrt[3]{3\ln(\eta_r) + \frac{3}{2}\left(\eta_{sp}\right)^2 - 3\eta_{sp}} \tag{5}$$

Deb and Chatterjee, 1969

$$[\eta] = \left[2\left(\eta_{sp} - \ln\left(\eta_r\right)\right)\right]^{\frac{1}{2}} c^{-1} \tag{6}$$

Solomon and Ciută, 1962

(7) $[\eta] = \frac{1}{2c}\left[\eta_{sp} + \ln(\eta_r)\right]$ Ram-M.-R. and Yassen, 1986

the relation provided by Solomon and Ciută (Eq. (6)) gives acceptable errors between 1 and 15% (Curvale and Cesco, 2009), and for this reason is used in the present work.

## 2.6. Objectives

The primary objective of this project is to develop and optimize an efficient extraction process for the in-house production of a key component used in some of the company's products. This involves a comprehensive approach that combines an in-depth review of existing scientific literature with a series of targeted laboratory experiments. The goal is to determine the optimal extraction conditions for isolating the BoI from a fungal waste stream. Once the extraction process is well assessed at the laboratory level, an important step is to evaluate its scalability and provide a design for the industrial production.

In parallel, some analytical techniques must be identified, evaluated, and implemented to ensure the consistent characterization of the final product. These include methods to measure DCM, viscosity, and the presence and concentration of the target product. These analyses are critical for monitoring process performance and verifying product specifications. Moreover, the extraction method should be adaptable to different fungal biomasses, such as yeast or other alternative sources, allowing for the use of different feedstocks depending on availability and cost. Lastly, given the need for a range of viscosities at OC, a broader aim of the project is to design a robust and flexible process. This process should allow for the controlled manipulation of key operational parameters to achieve specific, predefined product viscosities. This will enable the production of materials adapted to meet diverse application requirements.

# 3. Materials and methods

## 3.1. Materials

The dry BM is supplied from [redacted], as a residue of the chemical industry. Commercial PB and BoI from BM are supplied from [redacted], while BoI from traditional source from Kraeber & Co GmbH and PB from traditional source from [redacted]. The chemicals DMAB, acetylacetone and sodium carbonate are from Sigma-Aldrich Chemie GmbH (Germany). Denatured ethanol (EtOH) 96% is acquired from TermoFisher Scientific (United States). NaOH from Akzo Nobel Industrial Chemicals GmbH, hydrochloric acid (HCl) 37% from Scharlau (MC scientifics, Australia), AcOH 60% in water from Vendico Chemical AB (Sweden) and L-(+)-lactic acid (LaOH) 80% from Jindan Europe (The Netherlands) are used in this work.

DI-H2O is produced in house with the water purification system PURELAB Prima DV35 from ELGA LabWater (United Kingdom). Tap water is used as a water source when not otherwise indicated. Two different centrifuges are used for different process scales namely Joanlab laboratory centrifuge LC500-6 (United States) and Sorvall legend RT. Herdolp magnetic stirrer MR2002, IKA ULTRA-TURRAX T25 digital homogeniser, and 1,000H electrode pHenomenal 221 are used throughout the study. UV-1280 from Shimadzu (Japan) and Perkin Elmer Spectrum 100 (Specac LTD, United Kingdom) with MIR TGS detector are used respectively as UV-Vis and ATR-FTIR spectrophotometers. The heating plates are IKA ret basic C.

The dry content is measured with a MBT 62-L moisture analyser (VWR, United States) and the desiccator where dry samples are incubated before FTIR analysis is from Haldenwanger, Germany. The viscosity is measured with a DV2T rotational viscometer from Brookfield AMETEK (United States).

## 3.2. Process method

The following section provides a detailed description of how the tests and analysis are performed in the eight main experiments (EXP) and the solubility test.

From EXP 1 to 8, the process is always performed as in Figure 3, namely composed of a lysis and alkaline step, acidic dissolution, and precipitation. Between the steps, centrifugation is used to separate the soluble from the insoluble fractions.

For reasons of clarity, in Figure 6 the names given to each stream of the process are pointed out. In particular the CP after lysis/alkaline step entering acidic dissolution is going to be referred to as CP1, and its corresponding SN as SN1, which is the first discarded fraction.

Similarly, CP2 is the centrifugation pellet after acidic dissolution (second discarded fraction) and SN2 the corresponding supernatant where BoI is dissolved.

Finally, CP3 and SN3 are the fractions generated by precipitation, where BoI is expected to be in CP3, which corresponds to the final product. When a final washing step is done to neutralize CP3, the final product takes the name of CP4.

All the washing waters are called WW followed by a number if subsequent washes are done.

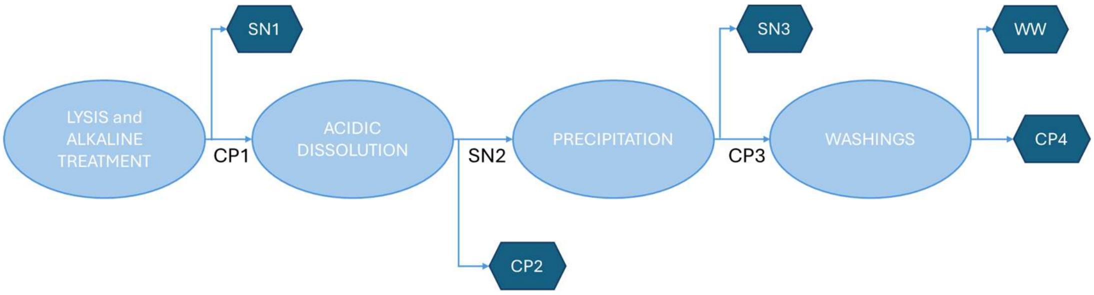

*Figure 6: Process scheme with focus on the acronym given to each fraction.*

### *3.2.1. First homogenization*

The cell lysis is performed with a homogenizer between 8000 and 15000 rpm and stirring with a propeller to prevent foaming.

In EXP1 lysis and alkaline treatment are done in one single step with 1 mol/l NaOH for 2 h at 90°C with 3% w/w BM.

In all the following EXPs, lysis and alkaline treatment are done separately. The first lysis step is done in water (except for EXP7, that is done with 0.5 mol/l NaOH) for 3 h at 40 - 70°C in 10% w/w BM.

### 3.2.2. *Homogenization in alkali*

The homogenization in alkali is done with stirring and homogenization at T between 90 and 115°C. Practically, it is performed adding NaOH (either in pellets or 8 mol/l solution depending on the desired final concentration) to the water lysate.

The final NaOH concentration is 4 mol/l in EXP2, EXP3 and EXP4, 19 mol/l in EXP5, 11.5 mol/l from EXP6 to EXP8.

### 3.2.3. *Washings*

The product of the homogenization steps is then centrifuged. The process is continued on the alkali insoluble fraction, namely CP1. In EXP1, EXP2 and EXP3.1 the pellet is directly neutralized via addition of acid. On the other hand, in all the following tests CP1 is washed until reaching a neutral pH before undergoing acidic dissolution.

In these washing steps tap water is added up to max centrifugal capacity, and the well stirred suspension is centrifuged. This process is repeated until the washing water reaches neutral pH.

### 3.2.4. *Acidic dissolution*

CP1 is then stirred at 60°C for 2 - 3 h in acidic solution.

The acid used in most of the EXPs is AcOH. Exceptions are in EXP3.1, EXP3.4 and EXP4 where LaOH is used instead, and EXP3.2 that is performed with HCl.

In the cases where CP1 is not washed, the amount of acid used is as much as needed to reach a pH of 3 - 3.5 measured with the pH-meter.

When CP1 is neutralized, acidic dissolution is done in 2% acid and 1:40 dry pellet to solution ratio. The 1:40 ratio is not satisfied by drying CP1, but measuring its dry content and assuming the rest is water.

Two tests of longer dissolution times were also done for 60 h at room temperature and for 24 h at 60°C as extra dissolution on CP2 from EXP5 and EXP8, respectively.

### *3.2.5. Precipitation*

SN2 generated from centrifugation of the acidic dissolution solution, is precipitated with NaOH solution (1 - 6 mol/l) until basic pH (7.5 - 14). The final precipitate obtained after this step is the target fraction.

### *3.2.6. Final washes*

CP3 is washed two to three times until reaching a neutral pH to remove residuals of salt, consisting mainly of sodium acetate/lactate.

### *3.2.7. Centrifugations*

All centrifugations were performed in two different centrifuges based on the scale at which the process was performed. In particular, the small-scale centrifuge has a max capacity of 300 ml and the large one of 2600 ml. All the centrifugations are done at max rpm of the corresponding centrifuge (4000 rpm for the small one and 4600 for the big one), for the time needed, which ranges between 10 min to 60 min.

### *3.2.8. Summary of all tested parameters.*

Table 1 contains a summary of all the main parameters from EXP1 to EXP8. The “scale” column reports the size of the batch and is related to the maximum capacity of the centrifuge. EXP8 is the combination of three smaller EXPs (8a, 8b and 8c) all performed with the same conditions.

In EXP4, the designed temperature of 90°C was not reached and the values reported show the actual range of T used. From EXP5, the maximum temperature of the range is reached at the moment of addition of the lye, then the designed T of 90 - 95°C is maintained with the heating plate.

The WW column contains the volume (in litres) of washing water that was necessary to reach a neutral pH measured with universal pH strips.

### *3.2.9. Solubility test*

The solubility properties of commercial BoI are tested in HCl, AcOH and LaOH in different concentrations with the purpose of determining the ranges where each acid could be used in the dissolution step.

With this purpose, different acid solutions ranging from 1% to 80% for LaOH, 1-60% for AcOH and 0.05-3.5% for HCl were prepared in 1ml volume. Commercial BoI is then dissolved by vigorously shaking starting from 1% (10 mg/ml) concentration and increasing it until the presence of a precipitate is confirming the reached max solubility.

|  | Scale | First homogenization |  |  |  |  | Homogenization in alkali |  |  |  |  | WW | Acidic dissolution |  |  |  |  |  | Precipitation |  |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
|  | liters | time | %w/w biom | T (°C) | [NaOH] | stirring | time | %w/w biom | T (°C) | [NaOH] | stirring | V (l) | acid | pH | %w/w acid | time(h) | stirring | T (°C) | [NaOH] | pH |
| EXP1 | 0.3 | / | / | / | 0 | / | 120 | 3.33% | 90 | 1 | no | / | AcOH | 3 | 14% | 2 | magnet | 60 | 2.5-5 | 14 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| EXP2.1 | 0.3 | 180 | 10% | 40 | 0 | propeller | 60 | 5% | 90 | 4 | propeller | / | AcOH | 3.5 | 44% | 2 | magnet | 60 | 5 | 9.7 |
| EXP2.2 | 0.3 | 180 | 10% | 40 | 0 | propeller | 60 | 5% | 90 | 4 | propeller | / | AcOH | 3.5 | 44% | 68 | magnet | RT | 5 | 11.1 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| EXP3.1 | 0.3 | 180 | 10% | 40 | 0 | propeller | 60 | 5% | 90 | 4 | propeller | / | LaOH | 3 | 56% | 2 | magnet | 60 | 2-5 | 8.7 |
| EXP3.2 | 0.3 | 180 | 10% | 40 | 0 | propeller | 60 | 5% | 90 | 4 | propeller | 0.36 | HCl | 0 | 1% | 2 | magnet | 60 | 2 | 11.8 |
| EXP3.3 | 0.3 | 180 | 10% | 40 | 0 | propeller | 60 | 5% | 90 | 4 | propeller | 0.41 | AcOH | 3 | 2% | 2 | magnet | 60 | 2 | 11 |
| EXP3.4 | 0.3 | 180 | 10% | 40 | 0 | propeller | 60 | 5% | 90 | 4 | propeller | 0.38 | LaOH | 2 | 2% | 2 | magnet | 60 | 2 | 11 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| EXP4 | 2.6 | 180 | 10% | 40 | 0 | propeller | 60 | 5% | 55-80 | 4 | propeller | 26.0 | LaOH | 2.8 | 2% | 3 | propeller | 60 | 5 | 7.5 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| EXP5 | 2.6 | 180 | 10% | 40 | 0 | propeller | 50 | 5% | 105 | 19 | propeller | 8.0 | AcOH | 3.5 | 2% | 3 | propeller | 60 | 5 | 8.5 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| EXP6 | 0.9 | 180 | 10% | 70 | 0 | propeller | 50 | 6.6% | 95-115 | 11.5 | propeller | 18.2 | AcOH | 3 | 2% | 3 | propeller | 60 | 6 | 14 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| EXP7 | 0.65 | 50 | 5% | 40 | 0.5 | magnet | 10 | 4% | 90-97 | 11.5 | propeller | 9.0 | AcOH | 3 | 2% | 3 | propeller | 60 | 6 | 14 |
|  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |  |
| EXP8a-b-c | 6.7 | 180 | 10% | 70 | 0 | propeller | 50 | 6.6% | 90-107 | 11.5 | propeller | 42.8 | AcOH | 3.5 | 2% | 3 | propeller | 60 | 6 | 9.5 |

*Table 1: Summary of the main parameters in all the experiments. Time is reported in minutes when not differently written and [NaOH] is mol/l. RT stands for room temperature.*

## 3.3. Analytical methods

This section provides an overview of how the analysis were performed, including the FTIR and viscosity measurements and the monomer assay.

### *3.3.1. FTIR*

The samples are dried in oven at 35 - 45°C for at least 24 h and left in the desiccator for 24 h before measuring the absorbance spectrum in the range 4000 $cm^{-1}$ - 500 $cm^{-1}$. All the spectra are normalized to their highest peak.

### *3.3.2. Viscosity*

For the measurement of viscosity, the final pellet is finely grinded in a mortar and dispersed in water, vigorously stirring for 30 minutes. To the dispersion, AcOH is added to get a final concentration of 1% dry CP4 (w/w%) and 1% AcOH. After 3 h incubation at 23°C, the viscosity is measured at 30 rpm.

### *3.3.3. Monomer assay by UV/VIS*

The acetylacetone reagent is prepared mixing acetylacetone with 0.5 mol/l sodium carbonate solution in 1:50 ratio. The solution is only stored for a maximum of 24 h and is kept in ice when not in use.

The DMAB reagent is prepared mixing 0.8 g DMAB with 30 ml ethanol and 30 ml 2 mol/l HCl. This reagent is stored in the fridge up to three months.

The samples to be measured are prepared taking an amount corresponding to a maximum of 2.5 mg M2 and adding water to a final mass of 405 mg and 80 µl (95 mg) concentrated HCl. The amount of sample is determined in two ways:

a) Assume that all the M2 present in the EXP (20% of the mass of BM) goes to the fraction of interest, calculate the corresponding concentration of M2, take a mass of sample corresponding to 2.5 mg.
b) Take a mass of sample according to Table 2, which is assessed after experimental observations under the assumption that in different EXPs the concentration of M2 in corresponding fractions is approximately in the same order of magnitude.

The so prepared sample solutions, which have a total mass of 500 mg and 2 mol/l HCl concentration, are hydrolysed in oven at 95°C for 24 hours.

Hydrolysed mixtures are added with 500 µl distilled water (DI-H2O) and 500 µl 2 mol/l NaOH. Then, 500 µl of this diluted and neutral solution is mixed with 500 µl acetylacetone reagent and incubated in oven @95°C for 10 min. The samples are then added with 500 µl of DMAB reagent before a final incubation of 30 min at

70°C. Finally, ethanol is added in 1:2 (sample to ethanol) ratio and each sample's absorbance at 530 nm is measured.

| TYPE OF SAMPLE | Mass of sample (mg) |
|---|---|
| Washing water, very diluted samples | 400 |
| Supernatants, average dilution | 150 |
| Wet centrifugation pellets (ex. CP2, wet CP4) | 40 |
| Dry and almost pure samples (ex. dry CP3, dry CP4) | 2.5 |

*Table 2: Mass of samples for the monomer assay.*

The calibration curve is done with commercial M2, PB and BoI by hydrolysing 5 mg/ml samples of each substance. The hydrolysate is then diluted with 1:1:1 DI-H2O, 2 mol/l NaOH and 2 mol/l HCl buffer in different ratios to make different concentration. The procedure is continued on each one of the calibration points as for the other samples.

# 4. Results and discussion

## 4.1. Solubility test

The results of the solubility test on commercial BoI are shown in Table 3.

**(a) HCl, 10 mg/ml BoI**

| **Acid concentration** | LV-BoI | HV-BoI |
|---|---|---|
| 0.05% (0.0137 mol/l) | ✗ | ✗ |
| 0.15% (0.0411 mol/l) | ✗ | ✗ |
| 0.30% (0.0823 mol/l) | ✓ | ✓ |
| 0.50% (0.1371 mol/l) | ✓ | ✓ |
| 1.00% (0.2742 mol/l) | ✓ | ✓ |
| 1.50% (0.4114 mol/l) | ✗ | ✗ |
| 2.50% (0.6857 mol/l) | ✗ | ✗ |
| 3.50% (0.9600 mol/l) | ✗ | ✗ |

**(b) HCl, LV-BoI**

| **Acid concentration** | 10 mg/ml | 15 mg/ml | 20 mg/ml | 30 mg/ml |
|---|---|---|---|---|
| 0.3% | ✓ | ✓ | ✗ | ✗ |
| 0.5% | ✓ | ✓ | ✓ | ✗ |
| 1% | ✓ | ✓ | ✓ | ✓ |

**(c) AcOH, LV-BoI**

| **Acid concentration** | 10 mg/ml | 15 mg/ml | 20 mg/ml | 25 mg/ml | 35 mg/ml |
|---|---|---|---|---|---|
| 1% | ✓ | ✓ | ✓ | ✗ | ✗ |
| 2% | ✓ | ✓ | ✓ | ✓ | ✗ |
| 60% | ✓ | ✓ | ✓ | ✓ | ✓ |

**(d) LaOH, LV-BoI**

| **Acid concentration** | 20 mg/ml | 25 mg/ml | 35 mg/ml |
|---|---|---|---|
| 1% | ✓ | ✗ | ✗ |
| 2% | ✓ | ✓ | ✗ |
| 10% | ✓ | ✓ | ✓ |
| 50% | / | / | ✓ |
| 80% | / | / | ✓ |

*Table 3: Results of the solubility test in (a) and (b) HCl, (c) AcOH and (d) LaOH. LV and HV respectively stand for low viscosity and high viscosity.*

Since the two commercial BoIs with different viscosities show similar solubility properties in the first dissolution test in HCl, the following samples are done only on the lower viscosity one. BoI results soluble in hydrochloric acid only in the range of concentration between 0.3% and 1% (w/w%). Within this scope, the solubility increases from 15 mg/ml to 30 mg/ml with increasing acid concentration. The solubility in AcOH and LaOH results not having limits of acid concentration from 1% to respectively 60% and 80% (max concentrations of these acids available in-house).

## 4.2. Monomer assay

The calibration curves made with commercial M2, and BoI are shown in Figure 7. The fact that the two lines do not coincide is relatable to the incomplete hydrolysis of the polymer. This phenomenon can be explained by its insolubility in the hydrolysis medium, causing it to precipitate and not completely hydrolyse in 24 h of incubation in the oven.

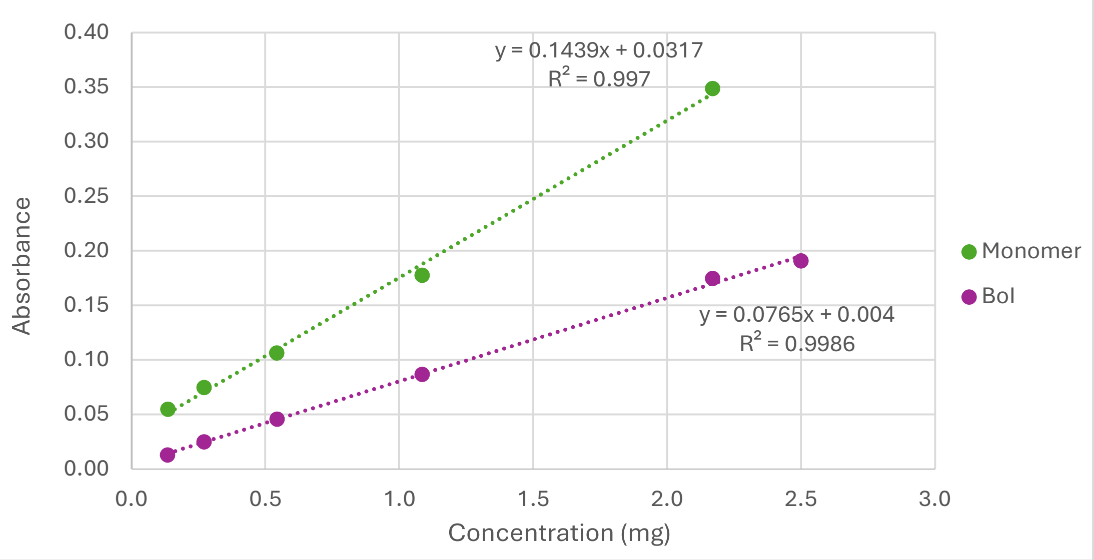

*Figure 7: Calibration curves made with commercial M2 and BoI.*

Using only the M2 curve would cause underestimation of the concentration in case a sample does not completely hydrolyse as it happens for the commercial BoI. On the other hand, the use of the only BoI curve would lead to overestimation of the detected concentration in the case of a completely hydrolysed sample.

To deal with that, the concentration of monomer is not uniquely extrapolated from one of the two curves, but the graph is used as a calibration range. The samples where the monomer is expected to be in a low molecular weight (such as SN1 and the washing waters) are estimated from the monomer curve, while the ones where the monomer should be present in a polymeric form (such as final pellet or CP2) refer to the BoI curve.

The monomer assay is initially performed on untreated BM with results reported in Table 4. The monomer detected in the BM is assumed to behave as a polymeric material, meaning that the concentration is calculated with the BoI calibration curve. The result of 11.56% is in line with the values available in literature.

| | **Mass of sample (mg)** | **Absorbance** | **Monomer (mg)** | **Content (%)** |
|---|---|---|---|---|
| BM (dry) | 9.5 | 0.088 | 1.098 | 11.56% |

*Table 4: Results of monomer assay on untreated BM.*

## 4.3. Results summary

In table 5, a summary of the results of all the main EXPs is reported.

The yield column is to be intended as a ratio between the amount of M2 detected in the product divided by the mass of BM used. For this reason, this value could at most correspond to approximately 11.6%. If one wants to calculate the yield with respect to the max expected monomer in the BM, the values reported should be divided by 11.6%.

The % in SN1&WW and % in CP2 columns, respectively correspond to the percentage of monomer that are detected in the first supernatant and washing waters (alkali soluble fraction) and the one detected in the pellet after acidic dissolution, namely the acid insoluble material. These two values are reported because they give an important insight of how the lysis/alkali step worked.

Specifically, having a large fraction of M2 lost in the first supernatant and washing waters implies that the alkaline conditions are too harsh, and a non-negligible fraction of the PB/BoI has depolymerized to the point where it gets soluble in water and alkaline solution.

The fraction lost in CP2, on the other hand, is a polymer that either did not have enough CM or is still bound to the insoluble SB. Both these cases imply that the alkaline step was not efficient enough. Moreover, the fraction lost in CP2 could be related to the efficiency of the acidic dissolution step: either a solution where BoI is not sufficiently soluble (wrong pH range or wrong acid), or a solution over the solubility limit where decreasing the concentration of solids would increase the yield (reduce these losses).

The final product is characterized with the monomer assay, whose result can be seen in the “purity” column, in the FTIR, where it is mainly compared with commercial PB and BoI (last column) and with a dissolution test. The dry final pellet is dissolved in 2% AcOH in a 1% ratio (pellet to solution) and vigorously shaken or stirred. If the dissolution is complete (as it should), the “dissolving” column reports a “yes,” otherwise a “no.”

The purity and the losses % in SN1 and WW and in CP2 reported in Table 5 are obtained from the monomer assay. In particular, the percentages are to be intended as the ratio between the amount of M2 in that fraction and the total detected. The fact that %in CP2, %in SN1&WWs and Yield/0.1156 do not always add up to exactly 100% can be attributed both to product losses during the process and to experimental variability of the method. It should be noted that not all outgoing fractions are consistently analysed in the assay, particularly with respect to WWs. This could lead to an underestimation of the losses and partial shift of the percentages in CP2 and SN1&WW. However, the yield column remains unaffected since it refers only to the BM used (and not to a “total M2 detected” in the assay). Overall, the discussion concerning CP2 and SN1&WWs is mostly qualitative and thus remains valid even if the reported values differ slightly.

| | **Yield** | **Purity** | **Dissolving** | **%in SN1&WWs** | **%in CP2** | **FTIR** |
|---|---|---|---|---|---|---|
| **EXP1** | 0% | / | / | 26.4% | 73.5% | / |
| | | | | | | |
| **EXP2.1** | 0% | / | / | 19.3% | 80.7% | / |
| **EXP2.2** | 0% | / | / | 17.2% | 82.8% | / |
| | | | | | | |
| **EXP3.1** | 1.4% | 1.8%(wet) | No | 9.3% | 74.3% | / |
| **EXP3.2** | 0.1% | 0.5%(wet) | No | 4.2% | 95.4% | / |
| **EXP3.3** | 1.9% | 3.5%(wet) | No | 2.8% | 90.6% | ≈PB |
| **EXP3.4** | 8.0% | 12.3%(wet) | No | 2.6% | 63.3% | ≈PB |
| | | | | | | |
| **EXP4** | 1.6% | 85-100% | No | 23.1% | 68.7% | BoI |
| | | | | | | |
| **EXP5** | 1.2% | 70-90% | Yes* | 68.9% | 22% | ≈BoI |
| | | | | | | |
| **EXP6** | 0.4% | 90-100% | Yes | 66.7% | 31% | BoI |
| | | | | | | |
| **EXP7** | 3.1% | 90-100% | Yes | 50.4% | 44.0% | PB |
| | | | | | | |
| **EXP8** | 1.0% | 80-100% | Yes** | - | 20-30% | impure |

*Table 5: Summary of results from EXP1-8. * Contains visible insoluble impurities. ** Only part of the produced fraction dissolved.*

Detailed analysis and interpretation of each of these results is reported in the following sections.

## 4.4. Washing steps

It can be seen that in EXP1 and 2, where the alkaline pellet is directly neutralized with AcOH, no final pellet is produced, resulting in a yield of 0%.

This phenomenon is attributed to a salting-out effect that happens because of the high concentration of sodium acetate, not allowing dissolution of BoI even at low pH. Consequently, the product is lost in CP2.

Moreover, when a high concentration of NaOH is used in the first step (as required to improve CM), the neutralization of all the lye without washing requires an exaggerate amount of acid, that is not environmentally and economically sustainable.

It is for these two reasons that the washing steps are introduced in all the following EXPs.

## 4.5. Acidic dissolution

An exception to the washing trend occurs in EXP3.1 where even without the washes a final pellet is present. This product though is not sufficiently pure if compared to the other fractions from the same EXP (the purity here is reported in wet conditions but can be compared to the ones resulting in EXP3.2, 3.3 and 3.4 since an assumption of similar dry content can be done).

This behaviour is not easily explained but can be attributed to the use of LaOH instead of AcOH. In general, LaOH seems to show wider but less selective solvent abilities as can be confirmed comparing the yields of EXP3.3 and 3.4. In both cases the final product has a PB-looking FTIR spectrum, but a different yield, that is higher with LaOH than AcOH. Practically, it is expected that a certain amount of low molecular weight PB could dissolve in acidic conditions (keep in mind that the DCM of PB is not necessarily 0, but everything between DCM0 and DCM50 is defined as PB; moreover, both M2 and M1 are soluble in water), but the higher yield in EXP3.4 shows that LaOH is better than AcOH at dissolving PB. This is an unwanted property, since the desired product is pure BoI, and not a mixture of BoI and PB.

It is for this reason that AcOH was chosen for the acidic dissolution step.

## 4.6. Homogenization in water and in alkali

The 3h homogenization in water step is introduced from EXP2 to keep at least the same homogenization time while reducing the chemical harshness of the alkaline step to decrease the losses in SN1&WWs. This modification is effective in that regard but results in an equivalent increase in the fraction lost in CP2.

Based on the tests conducted, it is difficult to identify any clear effect of this step on the process outcome. However, since homogenization is not expected to negatively impact on the product, as mechanical shear under the applied conditions is unlikely to induce significant depolymerization, the step is confirmed in all the subsequent EXPs. In phase of optimization, it could be of interest to test the relevance of this step and consider its removal should it prove to have no important effect.

In EXPs3 and EXP4, the final pellet produced does not pass the dissolution test and most of the monomer is lost in CP2. These facts are relatable to a not efficient homogenization and alkaline step. In fact, it can both mean that the polymer is not freed up from the cell wall's structure, or that it is not chemically modified enough, implying that harsher conditions are necessary in the alkaline step.

For this reason, from EXP5 to EXP8 higher alkali concentration is tested in the homogenization step with the purpose of finding an optimum between the minimization of the losses in SN1&WWs and CP2 and for the production of a final pellet with the desired properties (solubility in acid and BoI-looking FTIR spectra). The concentration of 19 mol/l NaOH at 95°C is first tested on commercial PB giving complete dissolution of both the products of 50 min and 100 min treatment. Therefore, the same conditions of concentration and shorter time are applied on the fungal extraction process giving a soluble final product. However, such process gives 69% of M2 lost in the first supernatant and washing waters. Moreover, the final product contains visible non soluble impurities likely relatable to side-reactions happening in such extreme conditions.

As expected, the concentration of 40% caused slightly increased losses in CP2, but reduced in the washing waters, together with a purer final product whose FTIR spectra is comparable to that of a commercial BoI.

Novikov et al. (2021) affirm that the CM starts to occur from a concentration of NaOH of 6.1 mol/l. Below that concentration, the hydration of the electrolyte ions overcomes the total hydration boundary (TBH) not allowing the CM to occur. This phenomenon could explain the non-sufficient CM in the first EXPs where NaOH was used at most at a concentration of 4 mol/l.

In EXP from 5 to 8 the lye is added in pellets, causing the temperature to increase to the boiling point of the solution in the moment of addition. For this reason, the temperature is only maintained in the range 80 - 115°C with the help of a heating plate and no tests were done at lower temperatures.

## 4.7. FTIR spectra interpretation

The spectra of the final products from EXP3.3 to 8 are reported in Figure 8. In the same figure also commercial BoI from fungi, PB from traditional source and a PB-SB complex from fungi are present. Since the peaks in the FTIR spectra can shift as a function of impurities and the characteristics of the molecule (such as the DCM) and most of the peaks result from the overlapping of different functional groups ([redacted]), the identification of the molecules via FTIR is not straightforward. However, the correlation of some characteristic bands to the functional groups together with the comparison with standard products can be done and results useful in the cross validation of hypothetical composition of the samples. For example, in the spectra of CP3.3 and CP3.4 from EXP3 and CP4 from EXP7 a sharp peak at 1555 cm$^{-1}$ corresponds to the N-H bending and C-H stretching of Amide II and the double peak at 1620 - 1650 cm$^{-1}$ to the N-H vibration of Amide I ([redacted]). Both these bands are typically present in the PB. The double peak at 1620 - 1650 cm$^{-1}$ merge to one single band in BoI ([redacted]).

The broad band between 3000 cm$^{-1}$ and 3500 cm$^{-1}$ can be attributed to hydroxyl group of polysaccharides interactions and overlaps with the N-H stretching band appearing around 3270 cm$^{-1}$, whose intensity varies with the DCM ([redacted]). In PB, this group shows two bands in the 3100 - 3290 cm$^{-1}$ region ([redacted]), while in BoI this pair gets smoothened.

The region around 2900 cm$^{-1}$ represents the C-H aliphatic bond vibration of BoI (Munoz et al., 2015; [redacted]; [redacted]).

The stretching of the C-O-C glycosidic bond is shown by peaks between 1020 - 1100 cm$^{-1}$ ([redacted]).

It can be concluded that the products of EXP3.3, 3.4 and 7 are mostly composed of PB, which is firstly partially dissolved in acidic dissolution, but shows then insufficient solubility after precipitation. This could be due to a partial CM and low molecular weight of this polymer that allows its solubility in limited concentrations.

The product closer to pure BoI appears to be CP4-EXP4 whose final insolubility in the dissolution test can be attributed to SB impurities.

The final pellet of EXP6 shows sharp peaks at 2850 - 2950 cm$^{-1}$ that [redacted] ([redacted]) attribute to lipidic impurities presumably coming from the cell wall that contains 2 - 7% of fats (Johnston, 1965). However, the good CM and solubility of the final product are the reasons why the parameters from EXP6 are chosen for the larger scale test (EXP8), and for the industrial process design.

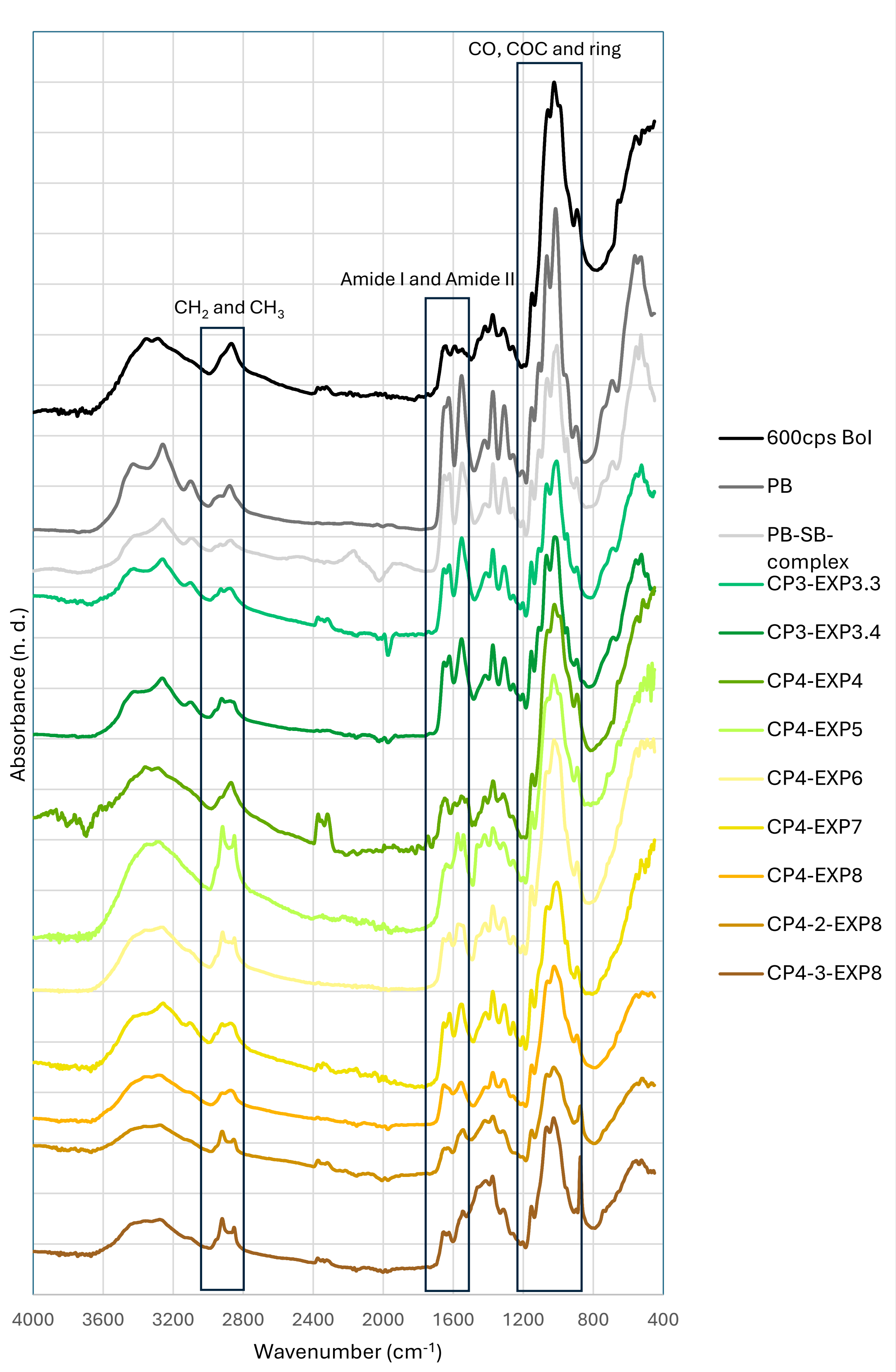

*Figure 8: FTIR spectra of the final product from exp3.3 to 8 and standards.*

The three different CP4 from EXP8 come from a test of subsequent dissolution of CP2. The washed CP1 is dissolved as usual, and the SN is precipitated giving CP4-EXP8. To increase the yield, the residual CP2 undergoes acidic dissolution again and the SN of this dissolution precipitated giving CP4-2-EXP8. Same is for CP4-3-EXP8 resulting from additional extraction of CP2 resulting from the second dissolution.

Seen the dependence of certain peaks on the DCM, wide literature is available about the use of the FTIR spectra to quantitatively estimate this property. In particular, Eq. (8), (9) and (10) are examples of equations that could be used for this purpose. The values of $A_n$ are the distance between the transmittance ($T = 10^{-A}$ where A is the output value from the machine when it is set in absorbance, at wavenumber of n $cm^{-1}$) and a certain baseline.

(8) $A_{1655}/A_{3450} = \frac{DCM}{115}$ Baxter et al., 1992

(9) $A_{1320}/A_{1420} = 0.3822 + 0.03133(1 - DCM)$ [redacted]

(10) $DCM = 100 - \frac{\frac{A_{1320}}{A_{3450}} - 0.03146}{0.00226}$ [redacted]

These equations are applied to commercial BoI with different DCMs, in order to assess their reliability. So far, no sufficiently consistent results have been obtained, resulting in a maximum $R^2$ of 0.57. For this reason, a precise quantification of the DCM is still not possible in-house, making external NMR analysis the only applicable method for this estimation (Czechowska et al., 2012). The details of this analysis are reported in Appendix 1.

## 4.8. Viscosity

EXP8 is the combination of three batches performed with the same parameters as EXP6. The viscosity (η) of the dry final pellet of this test is measured and the results are reported in Table 6.

| | **η (30 rpm)** | **η (20 rpm)** | **η (10 rpm)** |
|---|---|---|---|
| CP4-EXP8 | 7.6 | 6.6 | 6.6 |
| CP4-2-EXP8 | 4.6 | 4.8 | 4.8 |

*Table 6: Results of the viscosity test performed on the final pellets of EXP8. The viscosity η is in cps.*

This corresponds to a very low viscosity BoI, whose molecular weight can be estimated using Eq. (2), (3), (4), (6), where $\eta_r = \frac{\eta}{\eta_{1\%AcOH}} = \eta$ is the relative viscosity ($\eta_{1\%AcOH} = 1cps$) and $\eta_{sp} = \eta_r - 1$ is the specific viscosity.

Table 7 reports the so-calculated range of molecular weight. $K_1$ and $\delta_1$ are the constant in the case of DCM = 65 while $K_2$ and $\delta_2$ of DCM = 100.

|  | [η] (ml/g) | $K_1$ | $\delta_1$ | $K_2$ | $\delta_2$ | $M_{v1}$(kDa) | $M_{v2}$(kDa) |
|---|---|---|---|---|---|---|---|
| **CP4-EXP8** | 302.39 | $3.9*10^{-5}$ | 1.16 | 0.0164 | 0.8 | 892.7 | 233.7 |
| **CP4-2-EXP8** | 203.66 | $3.9*10^{-5}$ | 1.16 | 0.0164 | 0.8 | 634.3 | 146.0 |

*Table 7: Molecular weight estimation*

With unknown DCM, the molecular weight can range from 146 to 892.7 kDa, corresponding to the range from oligomeric to polymeric BoI.

It is important to consider that the resulting molecular weight is mostly an estimation since the reliability of the calculation of the intrinsic viscosity with a single-point method is not always guaranteed. In fact, [redacted] ([redacted]) report a molecular weight (MW) of 110–140 kDa, determined by gel permeation chromatography, for different BoIs with viscosities in the same range (3.1 to 6.2 cP).

## 4.9. OC-BioBinder® production

The final pellet of EXP8 is used in the production of one of the binders' formulations. The stiffness, strength and elongation are compared with the same product made with commercial BoI of the same category and one without any added BoI. The results with own produced BoI lays in the same range as the binder produced with commercial polymer, confirming that the BoI produced in-house with this method is suitable for the desired application.

# 5. Industrial process design

An important task of the project is to provide a comprehensive scheme of the industrial process, with the purpose of investigating the practical feasibility of such process and highlighting its key features and potential challenges and opportunities of improvement.

The industrial process is designed on the parameters of EXP6 and EXP8 since the wide experimental data available in relation to these conditions allows good estimation of the stream flows and other properties of the process. Moreover, the final product of EXP6 is the closest to BoI for purity detected in monomer assay and FTIR and solubility properties.

Figure 9 shows the scheme of the industrial process designed on a 250 kg BM scale. From the left side, the 5 m$^3$ tank is where the homogenization takes place. For the first three hours, the homogenization of the BM in water is done without addition of heat. The tank is jacketed and equipped with a propeller for stirring and reduction of foaming. The temperature can be measured with a temperature sensor and could reach a value of 35 - 45°C due to viscous dissipation. After the time of first homogenization, the pellets of NaOH are added to reach a final concentration of 11.5 mol/l. The dissolution of NaOH releases heat according to Eq (11).

$$(11) \qquad \Delta H_{diss} = n_{NaOH} * \Delta\widetilde{H}_{diss}$$

The values of $\Delta\widetilde{H}_{diss}$ are reported in Table 8 as a function of the concentration.

It can be seen that for the concentration of 11.5 mol/l NaOH (corresponding to 34% w/w), $\Delta\widetilde{H}_{diss} = 35.56\ kJ/mol$ (assuming linearity in the range 30.7-35.7%).

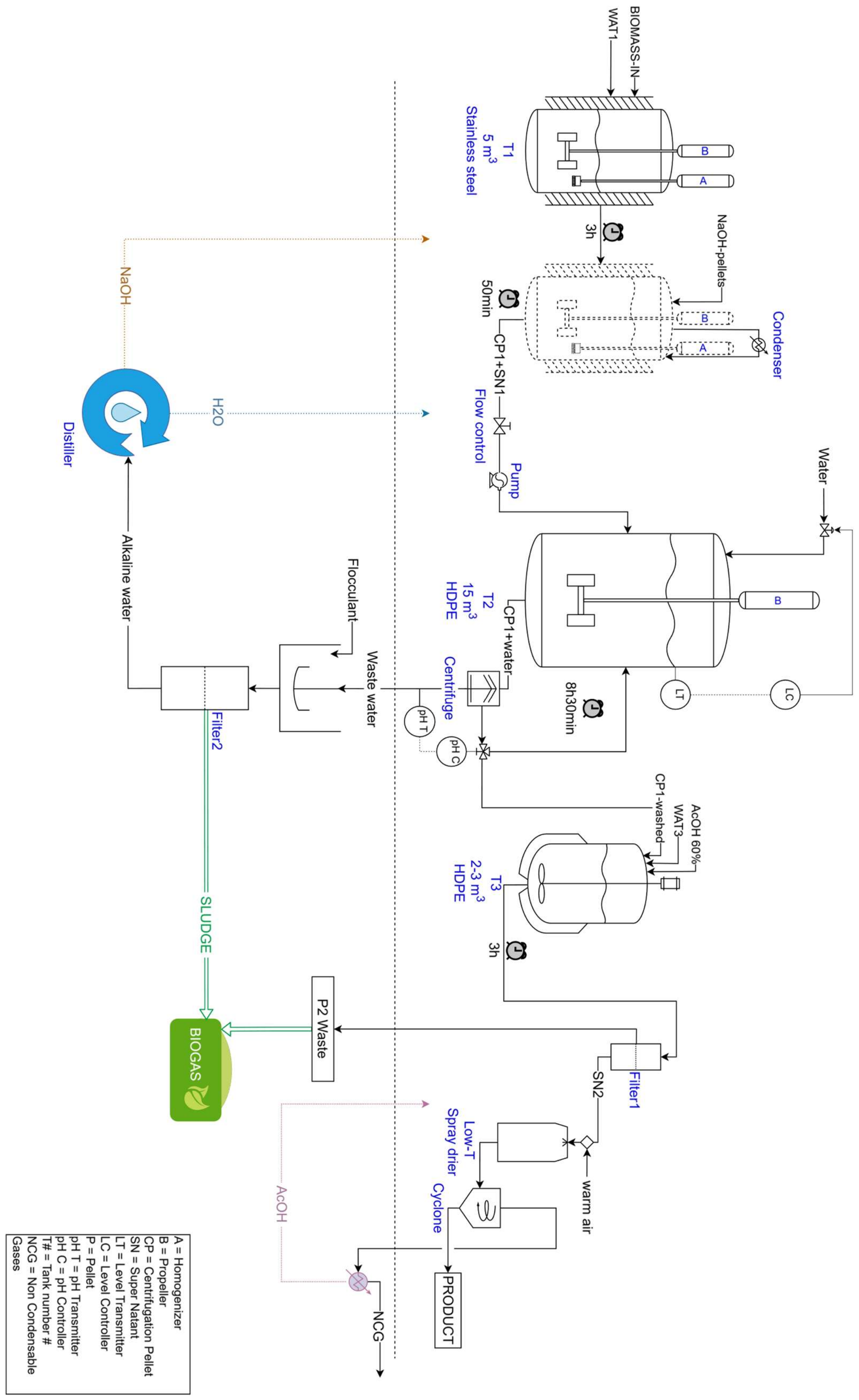

*Figure 9: Industrial process scheme*

| Concentration NaOH (mol/l) | Concentration of NaOH (%) | Heat of dissolution $\Delta\widetilde{H}_{diss}$ (kJ/mol) |
|---|---|---|
| 26.7 | 69.0% | 11.3 |
| 12.4 | 35.7% | 34.4 |
| 10.1 | 30.7% | 37.8 |
| 3.6 | 12.9% | 42.8 |
| 2.8 | 10.0% | 42.9 |

*Table 8: Heat of dissolution of NaOH in water function of concentration (Sergei, 2013).*

This results in 1159 MJ of heat released, that could make the solution reach a temperature of 138°C. For this reason, the tank is also equipped with a condenser for the vapours and their recirculation to the system. Detailed calculations can be found in Appendix 2.

The alkaline treatment lasts 50 min and is performed at the boiling temperature of the solution (approximately 110°C from lab observations) under stirring and homogenization. The tank and all the equipment in this step need to be built with a stainless-steel compatible with hot alkaline solutions that are corrosive to regular metals.

The lysed alkaline solution is then pumped to a 15 m$^3$ HDPE tank for the washes. An initial volume of water present in the tank of 12750 kg guarantees a final temperature of the solution of 47°C, which is safe with the use of a plastic tank. Detailed calculations can be found in Appendix 2. This tank can be equipped either with a propeller or with a homogenizer for the dispersion of the pellet in the solution, and a level control system for the water flow. A disk stack centrifuge with continuous solid discharge, separates and recirculates the partially washed pellet in the tank and discards the wastewater continuously. A pH control system allows to break the loop when the neutral pH of the discarded water confirms the sufficient removal of ions from the solution.

At this point, the pellet is sent to the final tank for acidic dissolution, together with AcOH and water to a final concentration of 2% acid and 1:40 solids to solution ratio. This step can be performed in a 3 m$^3$ stirred stainless steel tank heated to 60°C. For the separation of the insoluble pellet from the solution, a filtration system clarifies SN2 that is then sent to the final extraction step. This can be performed by spray drying if an acetate salt of BoI is tested to be acceptable and effective in the production of the binders. Alternatively, the separation can be done via alkaline precipitation, and successive wash of the product to remove the salts.

This process, with a yield of 1%, would produce 2.5 kg of dry BoI every 21 h. The schedule of the process is reported in Table 9. The most time-wasting step is the washing, where an approximate total of 48 tons of solution need to flow through the centrifuge.

| n° | ACTIVITY | | | | | time start | time end |
|---|---|---|---|---|---|---|---|
| **1** | Charging T1 with water | 0 | h | 10 | min | 00:00 | 00:10 |
| **2** | Weigh and add BM | 0 | h | 10 | min | 00:00 | 00:10 |
| **3** | First homogenization | 3 | h | 0 | min | 00:10 | 03:10 |
| **4** | Weigh and add NaOH | 0 | h | 10 | min | 03:00 | 03:10 |
| **5** | Homogenization in alkali | 0 | h | 50 | min | 03:10 | 04:00 |
| **6** | Pump from T1 to T2 | 0 | h | 10 | min | 04:00 | 04:10 |
| **7** | Washing | 8 | h | 30 | min | 04:00 | 12:30 |
| **8** | Charging T3 | 0 | h | 10 | min | 12:30 | 12:40 |
| **9** | Acidic dissolution | 3 | h | 0 | min | 12:40 | 15:40 |
| **10** | Filtration | 2 | h | 0 | min | 15:40 | 17:40 |
| **11** | Spray drying | 3 | h | 17 | min | 17:40 | 20:57 |

*Table 9: Timetable of the process*

Assuming to choose a centrifuge with 45 $m^3$/h solids handling capacity (such as MBUX 420 from Alfa Laval), with an oversizing factor of 1.5, requires 8.5 h of continuous running.

The process also produces 47 tons of alkaline wastewater and 650 kg of CP2 waste. The washing water is sent to an in-house distiller to be recycled in the process. With the introduction of a previous purification step on this water to remove cell debris, such as flocculation (Lee et al., 2014), part of the NaOH could be saved and recirculated to the system. To valorise organic content, the flocculated material is disposed of to a biogas plant for anaerobic digestion, together with the CP2 waste.

If spray drying is chosen for the final extraction, the condensation of the vapours permits the recycling of AcOH.

## 5.1. Consumptions

In terms of consumes, Table 10 contains the amount of chemicals that are needed in the process for each batch.

| Substance | Amount | Unit of measurement |
|---|---|---|
| Wet BM | 282.5 | kg |
| NaOH | 1287.9 | kg |
| Water | 46875.7 | kg |
| AcOH 60% | 47.2 | kg |

*Table 10: Chemical usage per batch*

The BM as supplied has a dry content of 88.5%. For this reason, the actual amount introduced in the process is corrected accounting for its wet content to a final dry BM treated of 250 kg. The amount of water necessary for the process is approximately 47 tons, of which 94% is used in the washing step. A total amount of 1.3 tons of NaOH is required for the alkaline step.

# 6. Conclusions

The present work is the result of the literature study and laboratory tests performed at OC about the extraction of a BoI from a fungal biomass. The final purpose is to commence the in-house production of a crucial element of their biobased binders.

The designed process consists of five steps among which are homogenization in water, alkaline treatment, acidic dissolution, and spray drying, resulting in a yield of 8.65% of the theoretical product present in the BM. The main losses are detected in the first supernatant and washing waters and the pellet after acidic dissolution. The fraction lost in SN1&WW is caused by depolymerization of the BoI, due to the harsh conditions in the alkaline treatment in terms of temperature, time, and lye concentration. The portion lost in CP2, on the other hand, is a fraction that did not sufficiently chemically modify and/or is still bound to the insoluble SB. Both these conditions would require harsher conditions in the alkaline step.

The industrial process, designed as a batch production, treats a total of 250 kg of BM (dry) in 21h, for the production of 2.5 kg of dry BoI that could be increased up to the theoretical max of 28.9 kg after process optimization. The washing water is recirculated in the system thanks to a distiller. The recycle of NaOH and AcOH needs to be further investigated but could allow the reduction of the consumption of chemicals, that is both environmental and economically advantageous.

The final product is a low viscosity BoI that can be tested for the use in the production of OC-BioBinder ® in combination with other commercial fractions.

The FTIR spectra of the final product revealed the presence of not identified impurities and fat residues in some of the EXPs, making it necessary to improve this aspect of the process, even with the help of additional analysis.

Further tests are necessary in order to increase the yield of the process, with a particular focus on the alkaline step conditions. Optimization of the designed process in terms of time and chemical consumption, could help to increase the potential profitability of the process.

# 7. Points of perspective

The present project aligns with the principles advised by the 2030 Agenda for Sustainable Development. Specifically, the use of alternatives to plastic and petrol-derived materials agrees with SDG14 about the preservation of the oceans by reducing plastic pollution. The innovative industrialization using side products, together with the waste treatment in the plant are in line with SDG9 and SDG12 promoting resource efficiency and waste prevention. Moreover, by reducing reliance on fossil-based resources and contributing to circular economy, the project supports SDG13 about climate action and aligns with targets such as 12.5 and 9.4, focused on minimizing environmental impact through sustainable innovation.

The designed process allowed the successful extraction of the BoI from fungal BM with the use of a compact workflow and common chemicals. Although, for reasons of time constraints, an optimal system is still not available both in terms of yield, that is limited in comparison with the theoretical available product, and properties of the extract.

A natural prosecution of the work could be to test more different parameters in the alkaline step, with the aim of reducing the losses both in SN1&WW and in CP2. In this regard, a possible strategy could be to investigate a supplementary alkaline treatment on the discarded CP2, for the further extraction of the product that is still insoluble in acid (either for its insufficient DCM or because of the SB contamination). This approach, if results in the possibility of additional production of BoI, could be combined with the minimization of the losses in SN1&WW leading to increased yield of the overall process. In the industrial scale this system would be applicable with the recirculation of CP2 to the first alkaline step, resulting in greater yield and reduced wastes.

It could also be of interest to test the relevance of the homogenization in water step, that was introduced for the improvement of the PB/BoI release from the cell wall, but its effects are still not confirmed.

The objective of the project to provide a robust process where the viscosity of the final product can be chosen a priori is not reached yet, having as available product only a low viscosity BoI. For this reason, the additional tests should also target the evaluation of the effects of the alkaline step conditions on the final product's viscosity.

Further investigation is also necessary in terms of analysis. The use of the calibration range in the monomer assay allowed a satisfactory assessment of the quantities despite the non-complete hydrolysis. However, for a more precise estimation of the product's purity, the use of a thermomixer instead of the oven in this step should be tested. Moreover, if this still does not allow acceptable efficiency, new analytical methods for the quality analysis should also be implemented. The use of FTIR or UV-vis spectroscopy for estimation of the DCM requires additional tests to reach satisfactory results, since at this point the only option is to send the samples for external examination.

An interesting aspect of the project is the investigation of the extraction of BoI from different fungal biomasses. The investigated BM is not the only one containing PB and BoI in its structure. Since the present method, intended as alkaline treatment and acidic dissolution-based, is based on the chemical properties of the BoI, is applicable to different biomasses. However, the exaction conditions in terms of times, concentrations and temperatures are related to the cellular structure and the DCM of the native product. For this reason, the direct generalization of the process to different biomasses may be impossible, requiring additional tests for the optimization to each specific biomass.

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# Appendix 1: DCM estimation via FTIR

For the investigation of the relationship between different absorption ratios and the DCM, three different equations are tested with 13 samples of commercial BoI with known DCM. The results of these predictions are stated in Table 11.

| **Actual DCM** | **Eq (8)** | **Eq (9)** | **Eq (10)** |
|---|---|---|---|
| 70.0 | 45.4 | 13.7 | 18.2 |
| 80.0 | 38.7 | 34.9 | 29.4 |
| 82.1 | 44.6 | -3.4 | 26.6 |
| 85.5 | 40.5 | 60.7 | 27.3 |
| 86.0 | 32.1 | 42.2 | 40.5 |
| 87.2 | 37.7 | 55.9 | 36.7 |
| 88.6 | 33.4 | 48.6 | 46.1 |
| 90.0 | 0.6 | 71.8 | 45.5 |
| 90.3 | 17.3 | 82.4 | 61.7 |
| 91.0 | 28.4 | 23.1 | 42.2 |
| 95.2 | 12.3 | 84.8 | 43.3 |
| 95.9 | 17.2 | 71.5 | 33.0 |
| 96.0 | 13.6 | 76.7 | 42.6 |
| **Mean error** | **59.7** | **36.5** | **49.6** |

*Table 11: Results of estimation DCM with the equations provided by Baxter and [redacted].*

The equations used for this purpose are:

(8) $DCM = \frac{A_{1655}}{A_{3450}} * 115$ (Baxter et al., 1992)

(9) $DCM = 100 - \frac{\frac{A_{1320}}{A_{1420}} - 0.3822}{0.03133}$ ([redacted])

(10) $DCM = 100 - \frac{\frac{A_{1320}}{A_{3450}} - 0.03146}{0.00226}$ ([redacted])

The peak at approximately 1655 $cm^{-1}$ corresponds to the amide I band, while 1320 $cm^{-1}$ appears specifically for M2 ([redacted]). Figure 10 shows the FTIR spectra of the BoI analysed, which had a DCM ranging from 70 to 96.

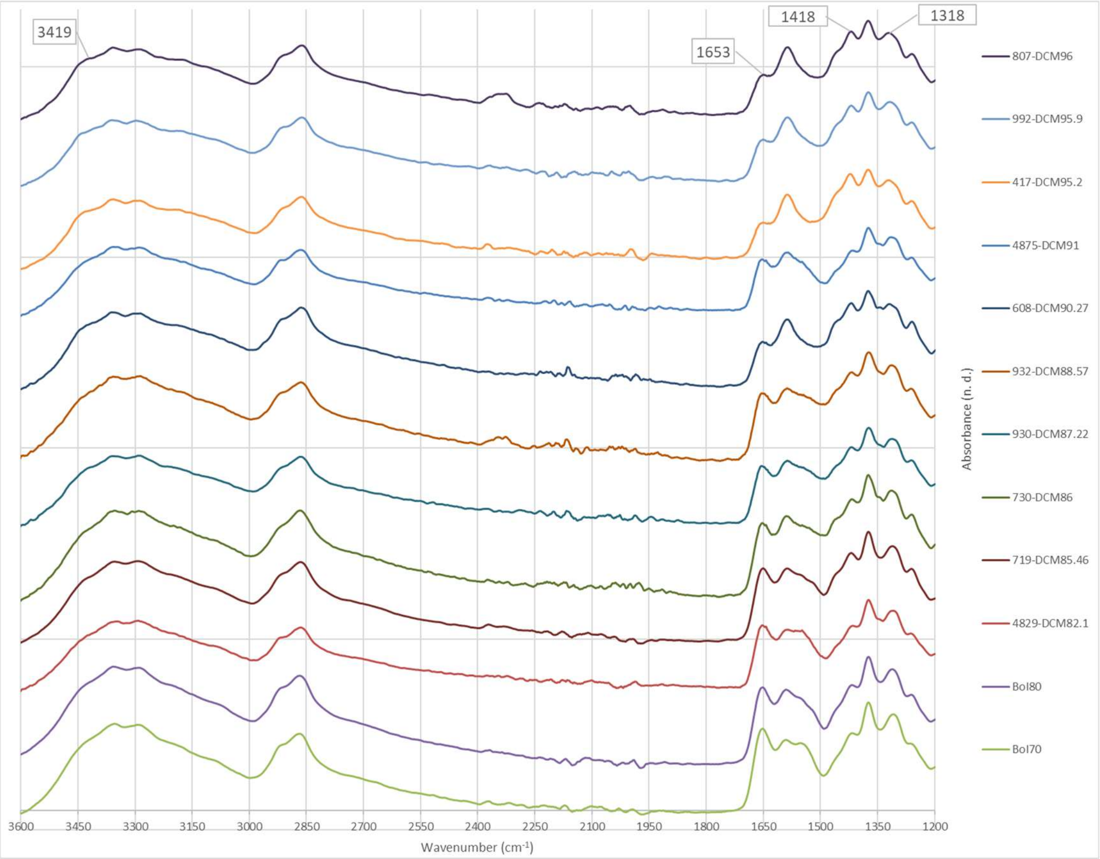

*Figure 10: FTIR spectra of BoI with different DCM focus on the of range of wavenumbers of interest for this analysis. The numerical code is an internal reference.*

The values of $A_n$ are calculated as a distance from the transmission at wavenumber n and a specific baseline. The valleys (peak in the transmittance spectrum) between which the baseline is drawn for each peak (valley in transmittances) is reported in Table 12.

| **n** | 1320 | 1420 | 1655 | 3450 |
|---|---|---|---|---|
| **baseline** | 1279-1343 | 1400-1459 | 1600-1700 | 1700-3600 |

*Table 12: Baseline used for each peak*

The three ratios are also plotted as a function of the DCM with the purpose of fitting the data in a new linear equation, resulting in a maximum $R^2$ of 0.573 corresponding to the ratio $\frac{A_{1655}}{A_{3450}}$. The plots are available in Figure 11.

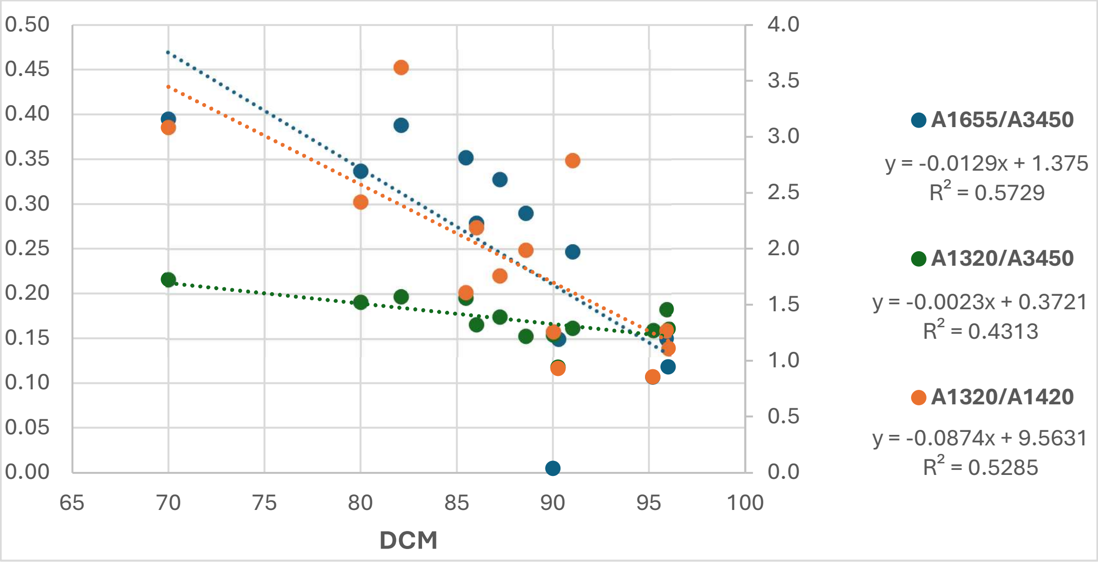

*Figure 11: Absorbance ratios as function of DCM.*

# Appendix 2: Heat calculations

## A2.1. Lye addition temperature

| | |
|---|---|
| $T_{IN} = 50\ °C$ | Temperature right before addition of NaOH |
| $m_{SOL} = \ 2500\ kg$ | Mass of solution before addition of NaOH |
| $m_{NaOH} = 1288\ kg$ | |
| $MW_{NaOH} = 40\ g/mol$ | Molecular weight NaOH |
| $\Delta\widetilde{H}_{soln} = -36\ kJ/mol$ | Heat of dissolution NaOH at 34% concentration (Sergei, 2013) |
| $c_p = 3.48\ kJ/kg°C$ | Specific heat of 34% NaOH solution (Bertetti, 1936) |

$$T_{fin} = T_{IN} - \frac{\Delta H_{soln} * m_{NaOH}}{MW_{NaOH} * (m_{NaOH} + m_{SOL}) * c_p} = 138\ \ °C$$

The addition of NaOH in a closed tank would make it reach a temperature of 138°C, with a potential final pressure up to 3.6 bar.

## A2.2. Temperature in water tank

| | |
|---|---|
| $T_{CP1+S} \quad = 115°C$ | Final temperature of alkaline solution (boiling point) |
| $T_{WW1} = 20°C$ | Temperature of the water |
| $m_{CP1+SN1} = 3788\ kg$ | Mass of alkaline solution |
| $m_{WW1} = 8962\ kg$ | Mass of water already in the tank |
| $c_{p,water} = 4.184\ kJ/kg°C$ | Specific heat of water |

| | |
|---|---|
| $c_{p,CP1+S} = 3.48\, kJ/kg°C$ | Specific heat of 34%NaOH solution (Bertetti, 1936) |
| $c_{p,FIN} = 4.178\, kJ/kg°C$ | Specific heat of CP1+SN1+WW1 |
| $\Delta\widetilde{H}_{soln} = -36\, kJ/mol$ | Heat of dissolution NaOH 34% concentration |
| $\Delta\widetilde{H}_{soln} = -42.9 kJ/mol$ | Heat of dissolution at infinite dilution (Sergei, 2013) |
| $\Delta\widetilde{H}_{dil} = -6.9\, kJ/mol$ | Heat released for the dilution of NaOH from 34% to 1% per mol of NaOH |

$$\Delta H_{dil} = \Delta\widetilde{H}_{dil} * \frac{m_{NaOH}}{MW_{NaOH}} = -222.196\, MJ$$

$$Q_{WW} = T_{WW1} * c_{p,water} * m_{WW}$$

$$Q_{CP1+S} = T_{CP1+SN1} * c_{p,CP1+S} * m_{CP1+S}$$

$$T_{FIN} = \frac{Q_{WW} + Q_{CP1+SN1} - \Delta H_{dil}}{m_{FIN} * c_{p,FIN}} = 46.71\, °C$$

The final temperature reached after mixing the alkaline solution and the first washing water is approximately 47°C.