Universitat Autònoma de Barcelona

# Optimization strategies for biopesticide production by fungal solid-state fermentation

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PhD in Environmental Science and Technology

# Optimization strategies for biopesticide production by fungal solid-state fermentation

PhD Thesis

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Bellaterra, Barcelona
2022

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Certifiquem:

que ARNAU SALA MARTÍ ha realitzat sota la nostra direcció el treball "**Optimization strategies for biopesticide production by fungal solid-state fermentation**", que es presenta en aquesta memòria i que constitueix la seva Tesi per optar al Grau de Doctor.

I, per a que se'n tingui coneixement i consti als efectes oportuns, firmem el present document.

Bellaterra, gener de 2022

Dra. Adriana Artola Casacuberta. Dra. Raquel Barrena Gómez

“Dona-li una altra volta”

Proverbi de millora i creixement personal

**Agraïments**

A la Universitat Autònoma de Barcelona per haver-me donat la oportunitat de realitzar la tesi doctoral i al Ministeri d'Economía i Competitivitat per aportar la financiació necessària (projecte CTM2015-69513-R).

A les meves directores de tesi,. Adriana Artola i Raquel Barrena, per tot el seguiment i l'ajuda dels 4 anys de tesi.

A la totalitat del grup d'investigació en compostatge (GICOM), encapçalat per Antoni Sànchez.

To Nicolai Vitt Meyling, for giving me the opportunity to perform part of my research in the Section for Organismal Biology (SOBI) research group in the University of Copenhaguen (KU).

Al Departament d'Enginyeria Química, Biològica i Ambiental, incloent tots els seus doctorands, professors, tècnics, estudiants i personal de neteja).

A los estudiantes que han participado en la investigación presentada en esta tesis (Silvana, Talia, Gonzalo i Melanie).

A les empreses Husk Ventures S.L. i Companyia Cervesera del Montseny per proporcionar els residus necessaris per realitzar les proves d'aquesta tesi.

Per últim, a la meva família per tot el suport que m'han donat sempre, i especialment durant aquesta important etapa de la meva vida.

**Agraïments extraoficials**

Hi ha etapes de la vida que et marquen. Escric aquestes paraules amb 30 anys al sac i encara sense data de defensa segura degut a l'atac informàtic que ha patit la UAB. Esperaré el meu torn amb paciència. Sé que per molt que tardi, no oblidaré el que he viscut. Així, mentrestant, tinc temps d'explicar i d'agrair tot allò necessari de la que ha estat, sense cap ombra de dubte, la millor etapa de la meva vida.

Vull començar dient que penso que he anat tard. Però no perquè hagi acabat la tesi en quatre anys (en una època on la majoria de tesis es fan en tres, si fos per mi n'hauria fet més i tot si me'ls haguessin pogut pagar). El motiu es precisament per l'edat. La majoria d'investigadors doctorats surten amb menys de 30 anys i el títol sota el braç. En un món tant competitiu, per desgràcia això pot ser considerat "anar tard". Però quan hi reflexiono bé, el que podria ser un problema esdevé una gran virtut. Perquè si hagués fet la tesi abans hauria passat per una experiència diferent amb altra gent. El que he viscut, ara ja no ho canviaria.

He de dir que des del principi, per tots els inputs i per les meves pròpies sensacions, des del primer moment vaig tenir clar que el meu "retorn" al Departament havia estat al grup correcte. Quan des de fora se't diu que has anat a "un grup molt maco" es que l'has encertat. Amb total sinceritat, abans de començar aquesta tesi, jo ni sabia el que era la fermentació en estat sòlid ni estava interessat en la sostenibilitat. Ara mateix, d'aquest segon concepte n'intentaré fer una carrera professional, aprofitant tot el coneixement que he obtingut a partir de treballar en el primer. Quan vaig llegir el tema de la meva tesi, vaig pensar alguna cosa com ara "sembla interessant" o "pot estar bé", però de segur no va ser res gaire més profund. Miro enrere i agraeixo que se'm donés la oportunitat des del GICOM. Aquest grup, en la seva totalitat, ha set molt més semblant a una família que a un grup de companys de feina. Treballar del que t'agrada envoltat de persones fantàstiques es impagable. Independentment de cap a on vagi el meu futur professional, crec que agrairé aquesta oportunitat tota la vida.

En aquest punt, vull expressar un agraïment especial cap a les meves tutores, Adriana i Raquel. No puc resumir acuradament tot el que m'han ensenyat durant aquests quatre anys, només vull dir que per a mi són un model a seguir a nivell professional. El plaer de treballar al vostre costat ha estat meu. Malgrat totes les vegades que hi he hagut

de donar “una altra volta”. També vull mencionar també a l’Antoni Sànchez, cap del grup, per tota la seva participació en aquesta tesi, malgrat no ser-ne el director.

Malgrat que jo feia uns anys que volia perseguir un doctorat (ni que fos per l’orgull de poder posar “Dr” o “PhD” a tot arreu), tothom qui ha fet un doctorat sap que el camí no es fàcil. D’entrebancs en forma de proves que no surten i cal repetir, caps de setmana que cal anar a treballar perquè els teus microorganismes així ho volen, escriptura d’articles inacabable perquè sempre es pot millorar donant-li “una altra volta”, revisions igualment interminables degut a múltiples rebuigs de les revistes... malgrat tot el temps que s’ha de dedicar, segueix sent una experiència que no canviaria. Mai de la vida. Ja que m’ha permès conèixer multitud de gent nova i fantàstica.

En aquest punt vull dividir les persones de qui penso parlar en dos grups. En essència, una part són els que van acabar el doctorat abans que jo i la resta els que, quan jo defensi, encara hi seran.

Esther, Pili, Nathaly, Golafarin i Cong Cong: a totes vosaltres que encara hi sou, molts ànims i molta sort. No podria apuntar aquí tots els moments que podem recordar, però si alguna cosa de cadascuna de vosaltres.

Esther, entre les hores de feina i de cotxe, potser ets amb qui he passat més temps. Ets una gran persona amb qui es pot confiar. Estic convençut que ens tornarem a veure i que ja tindràs el doctorat gairebé acabat (i amb molts articles).

Pili, has estat aquí pràcticament des del principi i fins al final. Sempre he pogut comptar amb tu quan ho he necessitat. Gràcies per ser-hi.

Nathaly, no perdis mai aquest bon humor i aquest somriure que tens, dones moltíssima alegria per allà on passes. Molts ànims i molta sort amb la tesi.

Golie, I am thankful of knowing you and teaching you the path of the fungi. I’m sure you’ll be able to achieve fantàstic results in your thesis.

Cong Cong, I appreciate your kindness and awareness, always ready to learn new things. Never lose your curiosity.

Laura, Elena, Pía, Paula, Alejandra, Mònica i Silvana, a totes vosaltres que ja heu agafat altres camins.

Laura, poder-te conèixer  treballar amb tu ha estat una gran sort. No tinc cap dubte que faràs grans coses a la vida, tant de bo més endavant ens puguem tornar a trobar.

Elena, tu si que t'ho muntes bé. Quan tornis, ni que sigui per poc temps, espero que trobem una estona per anar a la muntanya i m'expliquis totes les teves aventures en altres països.

Pía, tu risa es completamente inolvidable e insustituible. Te deseo toda la suerte del munto en tu retorno a Chile.

Paula, des que no parles amb mi segur que ja t'ha oblidat del català. La teva alegria, els teus refranys i cançons no s'obliden.

Alejandra, muchas gracias por invitarme a tu bioda, nunca te lo podré agradecer lo suficiente. Y sobretodo, por llamarme tu amigo. Mucha suerte a ti, Luis y Maria.

Mònica, molta sort amb la tesi a Dinamarca, tens molt talent i segur que et sortirà perfecte. Si mai hi torno, et vindré a veure de nou.

Silvana, porque para mi ser tu tutor ha sido mucho mas que eso. Fue un placer trabajar contigo y te deseo lo mejor en tu vida y en tu carrera.

A totes vosaltres, us considero no només les meves companyes sinó les meves amigues, i us trobo a faltar des que els nostres camins ja no estan creuats. Heu fet molt per mi, i desitjo almenys haver fet una petita part del mateix per a tots i cadascun de vosaltres. Tant de bo us pugui tornar a veure a totes en un futur, tant en l'àmbit professional com fora.

Hi ha molta més gent al Departament que mereixeria molt més que unes petites línies de recordatori en aquesta tesi. Especialment, vull recordar a la Rosi, tècnic de laboratori del Departament, amb qui hem tingut molt bona relació des del màster. I també vull agrair al Jordi Cairó, sota al tutela de qui vaig realitzar el meu treball de màster al mateix Departament, per l'ajuda que m'ha donat en tots els casos en què m'ha donat bones referències.

In addition, my acknowledgements would not be complete without a reference to the SOBI in the University of Copenhagen. Despite Covid-19, I had the extreme luck and pleasure to perform part of my research in this awesome group. Although it was short in time, I enjoyed knowing all of the people that work there and I wish I had stayed for more time. Hope I can meet with you all in the future. Special thanks to Nicolai Vitt Meyling for giving me the opportunity to join this group in a research stay.

Si escric tot això és, és clar, perquè ja estic a punt (en teoria) de dipositar i defensar la tesi. Per tant, aquest camí s'està acabant. Malgrat sento que està ja gairebé finalitzat (ja que fa uns mesos que no treballo al Departament) queda l'últim pas, i el que potser també serà l'últim adéu amb la gran majoria de persones de qui he parlat. La vida tira endavant però mai se sap, potser en algun moment futur puguem tornar a treballar plegats o a gaudir d'algun bon moment.

Amb tot aquest text, el que vull transmetre es que la millor manera de créixer, tant personalment com professionalment, es estar envoltat de bona gent. Grans persones que, sense saber-ho ni proposar-s'ho, et fan millorar dia a dia. Estar envoltat de tots ells ha estat la meva gran sort durant aquests últims anys.

A part però no menys important, hi ha dos agraïments més dels quals vull deixar constància:

En primer lloc, un agraïment molt sincer a tota la meva família i especialment als meus pares. És impossible resumir tot allò que us dec en poques paraules. Si he arribat fins aquí es en gran mesura gràcies a tot el que m'heu ensenyat i a tot el que heu fet per a mi, no puc fer més que expressar la més gratitud sincera per vosaltres.

I per últim, vull fer un agraïment especial i diferenciat per la Maria. Perquè si no t'hagués conegut, tant la tesi com tota la resta hauria estat molt més difícil. Moltes gràcies per tot. Has estat i segueixes sent una de les persones més importants de la meva vida.

**Thesis overview.**

This thesis focuses on the use of solid-state fermentation (SSF) as an approach to produce fungal biopesticides through the valorisation of solid wastes (agro-industrial wastes) as substrates. This study is developed in the framework of the project "Estrategias de optimización de procesos de obtención de bioproductos a partir de residuos orgánicos mediante fermentación en estado sólido (BIOPRO, CTM2015-69513-R)" and corresponds to the first work focused on fungal biopesticides performed in the research group. In particular, two different fungal strains presenting different biocontrol applications have been used in this thesis, *Beauveria bassiana* (BB) and *Trichoderma harzianum* (TH). Several agro-industrial residues have been tested as substrate for both fungal strains, with rice husk and beer draff as the most relevant. Much of the work has been focused on improving the packed bed bioreactor (PBB) configuration, one of the most promising yet least exploited SSF reactor outside of laboratory scale.

The first results block of the thesis corresponds to SSF parameters validation and optimization (Chapter 4) and substrate screening (Chapter 5). Optimization tests with both strains in 0.5 L SSF PBB batch system were performed using rice husk as substrate by means of design of experiments. Moisture, initial inoculum concentration, airflow and C/N ratio were identified as key parameters for process development establishing optimum values. When working with BB, 65-70% moisture, $5.5x10^6$ conidia $g^{-1}$dm inoculum concentration, 20 mL/min airflow, 25ºC temperature and 40 C/N ratio were found as optimum values. When working with TH, same values were obtained for most of the tested parameters except for moisture (55-60%) and C/N ratio (25-55). Mixing presented a negative effect on BB conidia production but was positive to THs' when performed at 24 or 48 h after inoculation. Robustness of the performed fermentation process was demonstrated through box-plots, establishing a highly probable conidia production range valid both for BB and TH when using rice husk as substrate ($5.0x10^8$-$1.3x10^9$ conidia $g^{-1}$dm). Substrate screening allowed the definition of more suitable agro-industrial wastes as substrates, serving as a basis for selection of residues to perform and scale-up fungal conidia production. PCA analysis showed different relevant parameters depending on the strain. When working with BB, relevant parameters (initial pH and air-filled porosity $AFP_R$) link to proper adaptation to the substrate to ensure fungal growth. When working with TH, relevant parameters (cumulative oxygen consumption, initial moisture and total sugar content) relate to potential substrate biodegradability.

The second results block of the thesis corresponds to fungal conidia production strategies and scale-up of the SSF process in PBB up to 22 L. In Chapter 6, sequential batch reactor (SBR) was tested as a strategy to produce fungal conidia in PBB using both BB and TH. Process scale-up is presented with two substrates: rice husk (as a way to overcome SSF scale-up drawbacks due to its naturally high $AFP_R$) and beer draff complemented with wood chips. SBR strategy was not successful using rice husk, neither with BB nor with TH, due to the presence of non-inoculated *Aspergillus niger* (AN) in the substrate, capable of withstanding autoclaving. When using beer draff, single batch strategy was still the preferred approach for BB conidia production. However, successful SBR was conducted using TH in a 22 L PBB, obtaining 5 consecutive batches presenting sustained conidia production at values close to $2.0x10^9$ conidia $g^{-1}$dm, despite showing relevant temperature differences between different reactor widths. When using beer draff, differences in performance observed between 1.5 L and 22 L allowed the definition of a minimum $AFP_R$ value of 80% when working with beer draff or similar agro-industrial wastes. Both substrates demonstrated uniformity throughout the packed bed, as both conidia production, moisture and pH at final fermentation time did not present significant differences within bed height, demonstrating the robustness of the fermentation. In Chapter 7, selected PBB fermentations are compared with tray bioreactor fermentation, incorporating chitinase activity measurement as an indicator of the biopesticide activity. Different behaviour was detected in tray bioreactor depending on the used strain: while conidia production was dependant on airflow distance but chitinase production was independent when working with BB, opposite behaviour was observed when working with TH. When comparing reactor configurations, relevant differences in conidia production were observed when working with BB, while they were not observed when working with TH, suggesting higher versatility of the TH strain. No significant differences in terms of mean temperature were observed between reactors, indicating an overall correct heat transfer.

Third results block presents product application and biocontrol potential of the products obtained from 22 L PBBs. For BB, biocontrol potential was tested against the pest *T. molitor*, both in larvae and adult stages. BB virulence was not detected against larvae stage. However, virulence was demonstrated against adult stage, as insect mortality increased with concentration. Higher virulence was detected with biopesticide obtained from beer draff fermentations in comparison to rice husk. However, virulence diminished in comparison to plate samples, indicating the need of a correct process optimization at

all levels (fermentation, purification and formulation) to maintain virulence after fermentation.

Overall, results presented in this thesis represent a step towards the use of PBB as an alternative approach to fungal biopesticide production using agro-industrial wastes as substrates by SSF.

**Resum de la tesi.**

Aquesta tesi es centra en l'ús de la fermentació en estat sòlid (FES) com a mètode de producció de biopesticides fúngics a través de la valorització de residus sòlids (agro-industrials) com a substrats. Aquest estudi s'ha desenvolupat en el marc del procés "Estrategias de optimización de procesos de obtención de bioproductos a partir de residuos orgánicos mediante fermentación en estado sólido (BIOPRO, CTM2015-69513-R)" i correspon al primer treball orientat a la producció de biopesticides fúngics portat a terme en aquest grup de recerca. Concretament, en aquesta tesi s'han utilitzat dues soques fúngiques que presenten diferents aplicacions en el camp del control biològic, *Beauveria bassiana* (BB) i *Trichoderma harzianum* (TH). Diversos residus agro-industrials s'han analitzat com a substrat per a les dues soques, sent clofolla d'arròs i bagàs de cervesa els més estudiats en aquesta tesi. Gran part de la feina ha estat enfocada a la millora del reactor de llit empacat (RLE) com a forma d'operació, ja que és un dels reactors més prometedors però menys explotats en FES fora de l'escala de laboratori.

El primer bloc de resultats correspon a l'optimització (Capítol 4) i a l'avaluació de substrats (Capítol 5). Les proves d'optimització amb les dues soques es van realitzar en RLE FES de 0.5 L utilitzant clofolla d'arròs com a substrat. Utilitzant BB, es van trobar els següents valors òptims de producció de conidis: 65-70% humitat, $5.5x10^6$ conidis $g^{-1}$ms concentració d'inòcul, 20 mL/min cabal d'aire, 25ºC temperatura i 40 ratio C/N. Utilitzant TH, es van obtenir els mateixos valors per la majoria dels paràmetres exceptuant la humitat (55-60%) i el ratio C/N (22-55). L'agitació del material va presentar efecte negatiu en la producció de conidis de BB però va ser positiu per a TH quan es practicava a les 24 o 48 h després d'inocular. La robustesa del procés de fermentació es va demostrar utilitzant gràfics de caixa, establint un rang altament probable de producció de conidis vàlid per BB i TH utilitzant clofolla d'arròs com a substrat ($5.0x10^8$-$1.3x10^9$ conidis $g^{-1}$ms). Els resultats de l'avaluació de substrats van permetre definir residus agro-industrials adequats per a la producció, servint com a base per la selecció de residus per a producció i escalat de la producció de conidis fúngics. L'anàlisi PCA va revelar diferent paràmetres rellevant en funció de la soca utilitzada. Respecte a BB, els paràmetres rellevants (pH inicial i porositat) estan relacionats amb una correcta adaptació al substrat per assegurar el creixement fúngic. Respecte a TH, els paràmetres rellevants (consum acumulat d'oxigen, humitat inicial i sucres totals) estan relacionats amb la degradació potencial del substrat.

El segon bloc de resultats correspon a estratègies de producció i escalat fins a 22 L del procés de FES. Al Capítol 6, l'estratègia de batch seqüencial (SBR) s'analitza com a estratègia de producció de conidis fúngics en RLE utilitzant tant BB com TH. Es presenta l'escalat del procés amb dos substrats: clofolla d'arròs (com a opció per evitar els possible inconvenients habituals de l'escalat de FES gràcies a la seva elevada porositat) i amb bagàs de cervesa amb estelles de fusta. L'estratègia de SBR no va ser exitosa amb aquest substrat degut a la presència d'*Aspergillus niger* (AN) al substrat no inoculat i capaç de resistir cicles d'autoclau. Utilitzant bagàs de cervesa, l'estratègia de batch també va ser la preferible en la producció de conidis de BB. En canvi, l'estratègia de SBR va resultar exitosa utilitzant TH en RLE de 22 L, aconseguint 5 batchs consecutius mantenint la producció de conidis a valors pròxims a $2.0x10^9$ conidis $g^{-1}ms$, malgrat obtenir diferències significatives de temperatura en l'amplada del reactor. Es van observar comportaments diferents segons l'escala utilitzant bagàs de cervesa, permetent definir un valor mínim de 80% de porositat en fermentacions de bagàs de cervesa o residus similars. Es va obtenir uniformitat al llarg de la columna empacada amb els dos substrats, ja que no es van observar diferències significatives respecte a l'alçada del reactor entre la producció de conidis, la humitat i el pH mesurats al final de la fermentació, demostrant la uniformitat i robustesa de la fermentació. Al Capítol 7, diversos RLE seleccionades son comparades amb fermentacions en reactor de safates, amb l'afegit de la mesura de l'activitat quitinasa com a indicador de possible activitat biopesticida. Es van detectar diferents comportaments en els reactors de safates en funció de la soca utilitzada: utilitzant BB, la producció de conidis va resultar ser dependent de la distància a l'entrada a l'aire però la de quitinases va resultar-ne independent, en canvi, TH va presentar el comportament oposat. Comparant totes les fermentacions, es van observar diferències rellevants en la producció de conidis en els resultats de BB, mentre que no es van observar en TH, suggerint major versatilitat de la soca de TH. No es van observar diferències significatives entre la temperatura mitjana dels diversos reactors, fet que indica correcta transferència de calor als reactors.

El tercer bloc de resultats correspon a l'aplicació del producte i al potencial de control biològic dels productes obtinguts en les fermentacions de 22 L en RLE. Per a BB, el potencial de control biològic es va provar contra la plaga *T. molitor*, tant en fase larval com adulta. No es va detectar virulència de BB contra la fase larval. Per contra, es va demostrar virulència contra la fase adulta, ja que la mortalitat va augmentar amb la concentració. Es va detectar virulència més elevada en el biopesticida obtingut a partir de

bagàs de cervesa en comparació amb clofolla d'arròs. Malgrat tot, la virulència va disminuir en comparació amb les mostres en placa, senyalant la necessitat d'optimització del procés en bloc (fermentació, purificació i formulació ) per mantenir la virulència després de la fermentació.

En general, els resultats presentats en aquesta tesi serveixen com un pas endavant en l'ús de RLE com a alternativa per a la producció de biopesticides fúngics mitjançant FES utilitzant residus agro-industrials com a substrats.

**Resumen de la tesis.**

Esta tesis está centrada en el uso de la fermentación en estado sólido (FES) como método de producción de biopesticidas fúngicos mediante la valorización de residuos sólidos (agro-industriales) como sustratos. Este estudio se ha ejecutado en el marco del proceso "Estrategias de optimización de procesos de obtención de bioproductos a partir de residuos orgánicos mediante fermentación en estado sólido (BIOPRO, CTM2015-69513-R)" y corresponde al primer trabajo orientado a la producción de biopesticidas fúngicos de este grupo de investigación. Concretamente, en esta tesis se han utilizado dos cepas fúngicas que presentan diferentes aplicaciones en el campo del control biológico, *Beauveria bassiana* (BB) y *Trichoderma harzianum* (TH). Varios residuos agro-industriales se han analizado como sustrato para las dos cepas, con la cáscara de arroz y el bagazo de cerveza como los dos mas estudiados en esta tesis. Gran parte del trabajo ha sido enfocado a la mejora del reactor de lecho empacado (RLE) como modo de operación, por ser uno de los reactores mas prometedores peró menos explotados en FES fuera de la escala de laboratorio.

El primer bloque de resultados corresponde a la optimización (Capítulo 4) y a la evaluación de sustratos (Capítulo 5). Las pruebas de optimización con las dos cepas se realizaron en RLE FES de 0.5 L con cáscara de arroz como sustrato. Con BB, se obtuvieros los siguientes valores óptimos de producción de conidios: 65-70% humedad, $5.5x10^6$ conidios $g^{-1}$ms concentración de inóculo, 20 mL/min caudal de aire, 25ºC temperatura y 40 ratio C/N. Con TH se obtuvieros los mismos valores para la mayoría de los parámetros, excepto para la humedad (55-60%) y la ratio C/N (22-55). La agitación del material presentó efecto negatico en la producción de conidios de BB peró fue positivo para TH cuando se practicó a las 24 o 48 h después de inocular. La robustez del proceso de fermentación se demostró con gráficas de caja, estableciendo un rango altamente probable de producción de conidios válido para BB y TH usando cáscara de arrçoz como sustrato ($5.0x10^8$-$1.3x10^9$ conidis $g^{-1}$ms). Los resultados de la evaluación de sustratos permitieron definir residuos agro-industriales adecuados para la producción, sirviendo como base para la selección de residuos para producción y escalado de la producción de conidios fúngicos. El análisis PCA reveló diferentes parámetros relevantes en función de la cepa usada. Respecto a BB, los parámetros relevantes (pH inicial y porosidad) están relacionados con la correctada adaptación al sustrato para asegurar el crecimiento fúngico. Respecto a TH, los parámetros relevantes (conusmo acumulado de oxígeno,

humedad inicial y azúcares totales) están relacionades con la degradación potencial del sustrato.

El segundo bloque de resultados corresponde a estratégia de producción y escalado hasta 22 L del proceso de FES. En el Capítulo 6, la estratégia de batch sequencial (SBR) se analiza como estratégia de producción de conidios fúngicos en RLE utilizando tanto BB como TH. Se presenta el escalado del proceso con dos sustratos: cáscara de arrçoz (como opción para evitar posibles inconveninetes habituales de la FES gracias a su elevada porosidad) y bagazo de cerveza con astillas de madera. La estratégia de SBR no tuvo éxito con la cáscara debido a la presencia de *Aspergillus niger* (AN) en el sustrato no inoculado y capaz de resistir ciclos de autoclave. Con bagazo de cerveza, la estratégia de batch fue preferible en la producción de conidios de BB. Sin embargo, la estratégia de SBR resultó exitosa usando TH en RLE de 22 L, consiguiendo 5 batchs consecutivos manteniendo la producción de conidios en valores próximos a $2.0x10^9$ conidios $g^{-1}$ms, a pesar de lasdiferencias de temperatura observadasr en la anchura del reactor. Se observaron diferentes patrones según la escala usando bagazo de cerveza, permitiendo definir un valor mínimo de 80% de porosidad en fermentaciones de bagazo de cerveza o residuos similares. Se obtuvo uniformidad en la altura de la columna empacada con los dos sustratos, puesto que no se observaron diferencia significarivas respecto a la altura del reactor entre la producción de conidios, la humedad y el pH medidos al final de la fermentación, demonstrando la uniformidad y la rebustez de la fermentación. El el Capítulo 7, varios RLE seleccionados son comparados con fermentaciones en reactor de bandejas, con el añadido de la medida de la actividad quitinasa como indicador de posible actividad biopesticida. Se detectaron diferentes patrones en los reactores de bandejas en función de la cepa usada: con BB, la producción de conidios resultó ser dependiente de la distancia de entrada del aire peró la de quitinasas resultó independiente, en comparación, TH presentó el comportamiento opuesto. Comparando todas las fermentaciones, se observaron diferencias relevantes en la producción de conidios en los resutlados de BB, aunque no se observaron en TH, sugeriendo mayor versatilidad de la cepa de TH. No se observaron diferencias significativas entre la temperatura media de los reactores, hecho que indica correcta transferencia de calor en los reactores.

El tercer bloque de resultados corresponde a la aplicación del producto y al potencial de control biológico de los productos obtenidos en las fermentaciones de 22 L en RLE. Para BB, el potencial de control biológico se testeó contra la plaga *T. molitor*, en ambas fases larvaria y adulta. No se detectó virulencia de BB contra la fase larvaria.

Sin embargo, se demosró virulencia contra la fase adulta, puesto que la mortalidad augmentó con la concentración. Se detectó virulencia mas elevada en biopesticida obtenido usando bagazo de cerveza en comparación con cáscara de arroz. A pesar de todo, la virulencia fue menor en comparación con las muestras en placa, señalando la necesidad de la optimización del proceso en bloque (fermentación, separación y formulación) para mantener la virulencia después de la fermentación.

En general, los resultados presentados en esta tesis sirven como un paso adelante en el uso de RLE como alternativa para la producción de biopesticidas fúngicos mediante FES usando residuos agro-industriales como sustratos.

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**Figure 8.6.** Adult *T. molitor* virulence test results a) total mortality and mycosis and b) survival evolution. P indicates plate samples (KVL or CECT strains), SSF indicates fermentation samples (CECT strain). Low concentration (L) corresponds to $10^6$ conidia/mL, high concentration (H) corresponds to $5x10^7$ conidia/mL.....

**Figure 8.7.** Examples of insect mycosis observed in dead T. molitor in virulence test. Left samples correspond to $1x10^6$ conidia $mL^{-1}$ and right samples to $5x10^7$ conidia $mL^{-1}$ a) KVL-P-L; b) CECT-P-L; c) CECT-SSF-RH-L; d) CECT-SSF-BDr1-L; e) KVL-P-H; f) CECT-P-H; g) CECT-SSF-RH-H; h) CECT-SSF-BDr1-H and i) CECT-SSF-BDr2-H.

## Tables summary

**List of abbreviations**

| Abbreviation | Definition | Unit |
|---|---|---|
| AF | Airflow | mL $min^{-1}$ |
| $AFP_R$ | Air filled porosity | % |
| AN | *Aspergillus niger* | |
| AP | Apple pomace | |
| BB | *Beauveria bassiana* | |
| BD | Bulk density | g $L^{-1}$ |
| BDr | Beer draff | |
| BG | *Burkholderia gladioli* | |
| BT | *Bacillus thuringiensis* | |
| C/N ratio | Carbon/nitrogen ratio | |
| CD | Conductivity | μs $cm^{-1}$ |
| CECT | Colección Espanyola de Cultivos Tipo | |
| CFU | Colony forming units | |
| COC | Cumulative oxygen consumption | $gO_2$ $kg^{-1}$dm |
| CP | Conidia production | conidia $g^{-1}$dm |
| CPr | Conidia productivity | conidia $g^{-1}$dm $d^{-1}$ |
| CQ | Conidia quotient | |
| DM (dm) | Dry matter | % |
| DoE | Design of experiments | |
| FAO | Food and agricultural organization | |
| FPU | Filter paper unit | |
| GI | Germination index | % |

| | | |
|---|---|---|
| IC | Inoculum concentration | conidia $g^{-1}$dm |
| $LT_{50}$ | Lethal time 50 | d |
| MC | Moisture content | % |
| $MC_I$ | Initial moisture content | % |
| MEA | Malt extract agar | |
| $O_2$ | Oxigen | % |
| OM (om) | Organic matter | % |
| OP | Orange peel | |
| PBB(s) | Packed bed bioreactor(s) | |
| PC | Principal component | |
| PCA | Principal component analysis | |
| PDA | Potato dextrose agar | |
| PP | Potato peel | |
| RF | Rice fiber | |
| RH | Rice husk | |
| SBR | Sequential-batch reactor | |
| SDAY | Saboraud dextrose agar + yeast extract | |
| SmF | Submerged fermentation | |
| sAF | Specific airflow | mL $min^{-1}$ $g^{-1}$dm |
| sOUR | Specific oxygen uptake rate | $gO_2$ $kg^{-1}$dm $h^{-1}$ |
| SF | Soy fiber | |
| SSF | Solid-state fermentation | |
| T | Temperature | ºC |
| TC | Time course | |

| | | |
|---|---|---|
| TH | *Trichoderma harzianum* | |
| TSC | Total sugar content | mg $g^{-1}$dm |
| WD | Whisky draff | |
| WS | Wheat straw | |

# Chapter 1

## Introduction

Part of this chapter was published in: Sala, A., Barrena, R., Artola, A., Sánchez, A., 2019. Current developments in the production of fungal biological control agents by solid-state fermentation using organic solid waste. Critical Reviews in Environmental Science and Technology. 49, 655-694.

## 1.1. Pest control: from chemical pesticides to biopesticides

Insect pests have always represented a major threat to crops all over the world. The coexistence of insects and plants has often adversely affected agriculture, rising the need to develop products for crop protection. For a long time, chemical pesticides were thought to be the best option against pests, as they significantly raised crop yields since the very beginning of their use (Stenersen, 2004). However, their drawbacks soon began to arose, as they pose a serious threat both to human health (due to their toxicity and mutagenic capabilities) and to the environment (as they are toxic not only for insect pests and weeds but also for the rest of non-targeted organisms, including both animals and plants (crops), spreading contamination through soil, air and water while conferring resistances against them to insects due to their mutagenic properties) (Bolognesi, 2003; Thakore, 2006; Sharma et al., 2014). All these problems support an urgent need to shift away from this tendency to obtain healthier crops while also protecting both environment and human health. This change is in turn supported by Integrated Pest Management (IPM) and backed up with increasing difficulties in discovering new synthetic pesticides (Pretty and Bharucha, 2015; Mascarin and Jaronski, 2016).

One of the proposed paths within IPM relies on applying biopesticides instead of chemical pesticides. Biopesticides involve the use of biological insecticides, mainly obtained by microbial fermentation, which represent a solid alternative to chemicals due to their harmless nature both to humans and to the environment and to the fact that they have not been shown to cause resistance when applied to insect pests (Allen and Levy, 2013; Smalling et al., 2013; Lai and Su, 2011). In 2017, the worldwide crop protection market was valued around 56.7 billion U.S. dollars, showing an increasing tendency from 2008 to 2018 with maximum values reached in the last years around 50-57 billion U.S. dollars, as presented in Statista (2021). As shown in Figure 1.1., total pesticides (including biopesticides) use was superior to 4.1 million tonnes worldwide by 2018 (from which nearly 1.4 million tonnes corresponded to China, almost 407k tonnes corresponded to the U.S. and 222k tonnes corresponded to Brazil on average), as presented by the Food and Agricultural Organization (FAO). In 2019, 5.2-8.2% of crops pesticides market was occupied by biopesticides (nearly 4 billion U.S. dollars), their growth is projected to outpace that of chemical pesticides, with variable growth per year between 10-20% (Marrone, 2019). Forecasts expect the biopesticides sector to grow worldwide from 4.3

to 8.4 billion USD in the 2020-2025 period at a Compound Annual Growth Rate of 14.7% from 2020 to 2025 (Markets and Markets, 2020).

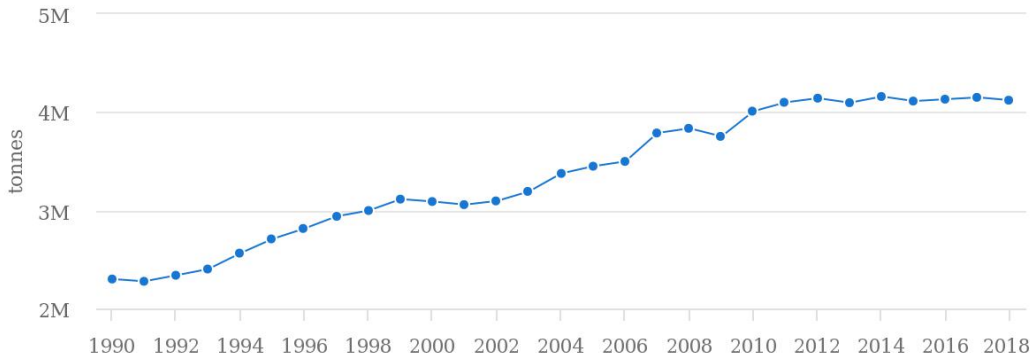



**Figure 1.1.** Total pesticides use evolution from 1990 to 2018. Obtained from FAO database in April 2021.

Global biopesticide products market based on the types of microbes used is shown in Figure 1.2. Bacterial products occupied 60% of the total biopesticide worldwide market as of 2015. Among them, *Bacillus thuringiensis* (BT) is the most used of all biopesticide products, since biopesticides derived from this microorganism can decompose quickly enough to be harmless against soil and water (Abubaker et al., 2015) while being highly specific towards the most common insect pests such as *Lepidoptera, Coleoptera* and *Diptera* (Mnif and Ghribi, 2015). Nevertheless, as shown in Figure 1.2., fungal bioproducts have also attracted global market's attention, having reached more than 25% of the total biopesticide worldwide market as of 2015, and have earned their place mostly due to their broader spectrum as well as to their production yields when compared to bacterial bioproducts (Copping and Menn, 2000).

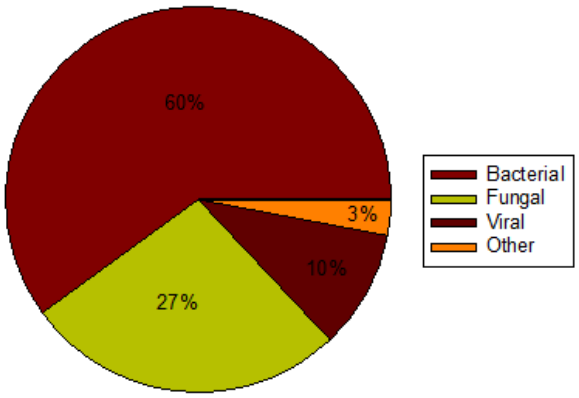



**Figure 1.2.** Global biopesticide market based on the types of microbes used. Data from Mishra et al. (2015).

## 1.2. Fungal biopesticides

Fungal biopesticides represent a solid alternative both to chemical pesticides and to BT based biopesticides. Fungi have evolved towards a parasitic lifestyle, becoming capable of infecting more than 1000 host species, which highlights their relevance in pest control, being crucial in the deaths of most insects and arachnids (Humber, 2008; Boomsma et al., 2014). As shown in Figure 1.3., research on fungal biopesticides has not stopped growing for the last 30 years, particularly since the 2000's, with more than 420 research documents published in 2020, according to Scopus. Among the large diversity of fungal biopesticides, two major groups must be distinguished due to their superior biocontrol capabilities, being entomopathogenic fungi (mainly represented by the genera *Beauveria*) and antagonistic fungi (mainly represented by the genera *Trichoderma*).

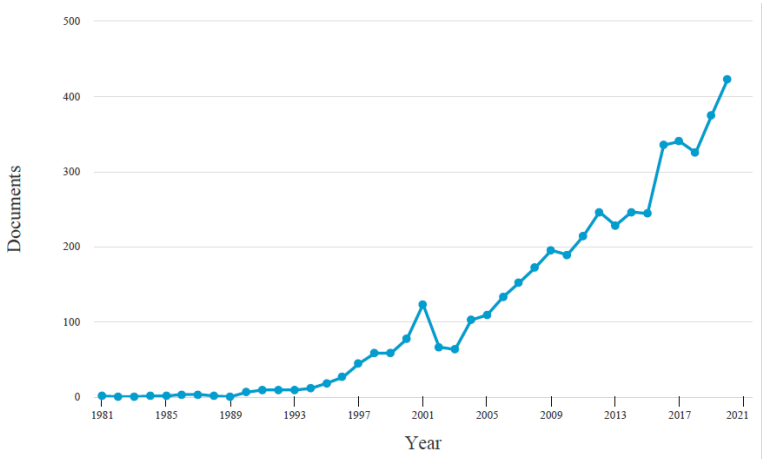



**Figure 1.3.** Fungal biopesticides research evolution from 1981 to 2020. Obtained from Scopus in April 2021.

### 1.2.1. Entomopathogenic fungi: *Beauveria* spp.

Entomopathogenic fungi are defined as fungi that can act as parasite of insects, killing or seriously disabling them. The majority of the fungal entomopathogens present an infective route based on contact with the insects' cuticle rather than based on ingestion, which is the most common for bacteria, viruses and other infective agents, effectively functioning as direct contact pathogens (Mascarin and Jaronski, 2016). Its infection cycle is schematized in Figure 1.4. After dispersion by wind, rain or insect vectors, asexual spores (namely conidia) attach to insect cuticle and germinate, forming a germ tube. The growing hyphae can breach the cuticle and penetrate the hemolymph, a nutrient-rich environment. Once there, the fungus undergoes a morphogenetic differentiation, going from filamentous growth to single-celled blastospores, which colonize internal tissues and evade the host immune system while also secreting toxic metabolites to support the colonization, culminating in the host's death. When that occurs, conidiophores emerge from the host's dead body after a few days, producing newly infective conidia (sporulation) to continue the pathogen's life cycle. (Mascarin and Jaronski, 2016). An extensive list of fungal genera which possess entomopathogenic properties was reviewed by Evans et al. (1997), from which the most relevant is *Beauveria* spp.

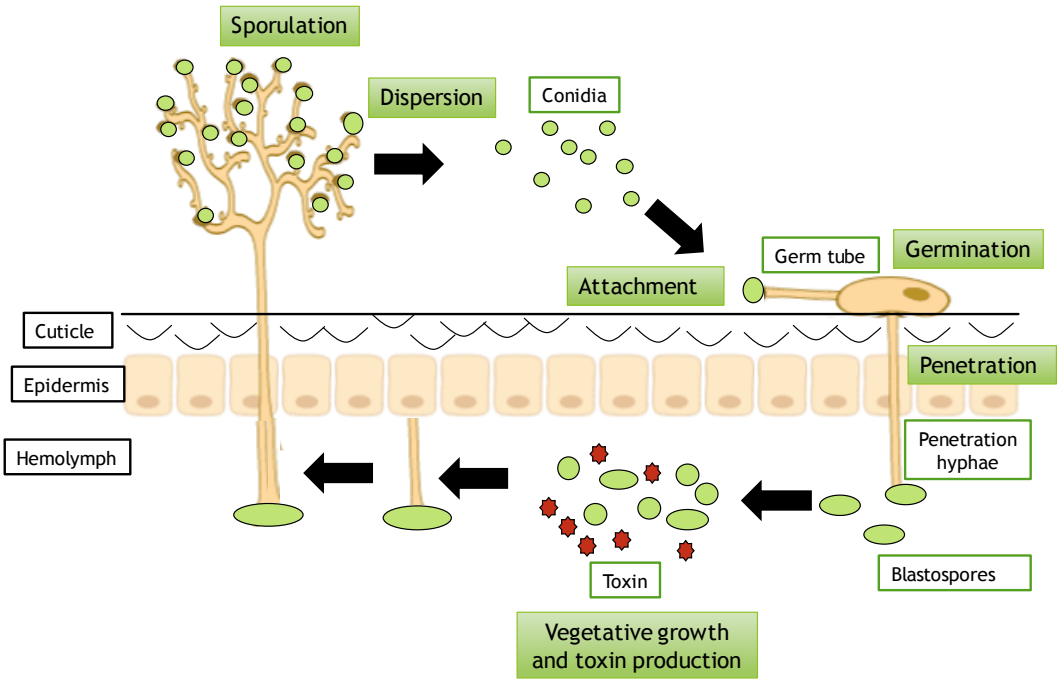



**Figure 1.4.** Schematic representation of the basic infection cycle in *Beauveria bassiana*. Adapted from Mascarin and Jaronski (2016).

The genera *Beauveria* is one of the most extensively studied entomopathogenic fungi around the world, with thousands of isolates documented (Rehner et al., 2011). It is pathogenic to more than 700 species of hosts, including various species in A*cari* and *Insecta* (Inglis et al., 2001; Zimmerman, 2007). Some fungal strains in the genus also present endophyte capabilities, being able to live inside several plants without harming them and allowing the colonisation of insects if they are susceptible to the fungal spores (Behie et al., 2012; Meyling and Eilenberg, 2007). Scopus search conducted in April (2021) retrieved almost 5300 documents related to the genus, almost 93% of which are research articles. The first one was published in 1912, with a growing tendency from the 1960s and especially from the 1990s to the present, with more than 300 documents (even surpassing 400 in 2020) published in each of the last three years. *Beauveria* spp. products are commercialized all over the world. Extensive lists of *Beauveria* spp. worldwide products, and particularly about *Beauveria bassiana* (BB), were reviewed by Faria and Wright (2007) and Mascarin and Jaronski (2016).

### 1.2.2. Antagonistic fungi: *Trichoderma* spp.

Antagonistic fungi are defined as fungi which affect or supress the normal activity of a plant pathogen, effectively serving as BCAs. Consequently, most antagonistic fungi are in symbiosis with the plant in which they live, triggering responses to benefit both fungi and plant (Verma et al., 2007; Martínez-Medina et al., 2019). Among all antagonistic fungi, the genera *Trichoderma* spp. have gained wide acceptance as effective BCA.

*Trichoderma* spp. are widely known for their antagonistic activity against several soil phytopathogens, involving both fungi, bacteria and invertebrates, however, its effect against non-fungal species is much more occasional (Verma et al., 2007). Srivastava et al. (2016) summarised all of *Trichoderma* spp. biological control mechanisms, which are: (i) induced systemic resistances against plant pathogens, (ii) fungistasis (resistance to solid compounds which inhibit fungal growth), (iii) production of cell wall degrading enzymes (some of which are key in lysis of cell wall of pathogenic fungi), (iv) competition with other fungi (*Trichoderma* spp. can rapidly colonize the roots and benefit from its nutrients), (v) mycoparasitism of other fungi and (vi) production of secondary metabolites (mainly enzymes, which has been widely exploded at industrial level to obtain several enzymes like cellulases, hemicellulases or proteases among others)

(Schuster and Schmoll, 2010; Verma et al., 2007). Processes of mycoparasitism, competition and metabolites production after mycelial growth around the rhyzosphere are well documented and exemplified in Figure 1.5. Notably, its use has been greatly successful against soil-borne diseases for which no resistant sources have been identified in plants (Sharma et al., 2014).

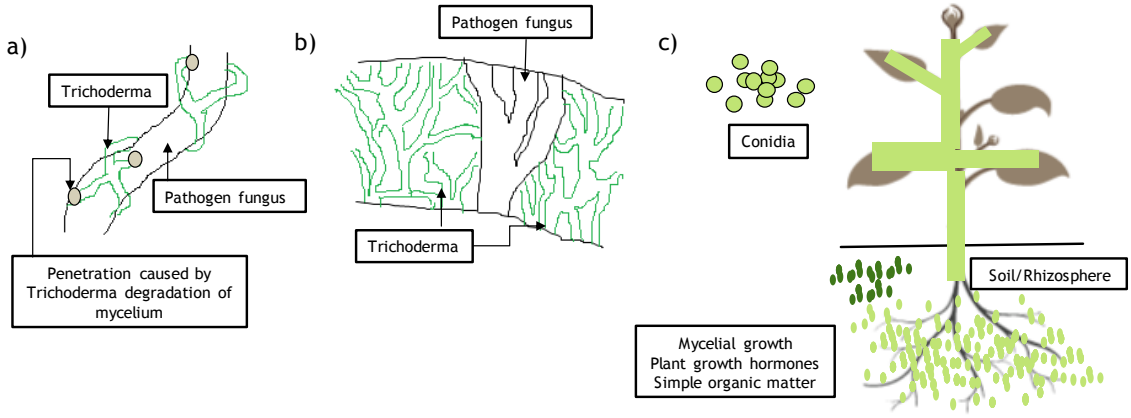



**Figure 1.5.** Principal antagonistic activities presented by *Trichoderma* spp: mycoparasitism (a), competition (b) and mycelial growth around plant rhizosphere coupled with metabolites production (c). Adapted from Verma et al. (2007).

Although their endophytic capabilities have not been studied in depth as those of *Beauveria* spp., some works have already presented *Trichoderma* spp. as an endophyte, such as Glare et al. (2012). A Scopus search performed in April 2021 retrieved more than 18500 documents related to the genus, more than 90% of which are research articles. The first one was published in 1897, showing a growing tendency as the one showed by *Beauveria* spp., albeit having higher absolute number of publications, with higher growth observed specially from the 2000s to the present, with more than 1250 documents published in 2020. As presented by Verma et al. (2007), *Trichoderma* spp. based BCAs are available in the market, even though a vast majority of them are promoted as soil enhancers or growth promoters. Among all *Trichoderma* species, when regarding BCA capabilities, *Trichoderma harzianum* (TH) is considered as the most effective (Gao et al., 2002).

## 1.3. Fungal biopesticides production by solid-state fermentation

### 1.3.1. Solid-state fermentation and submerged fermentation comparison for fungal biopesticide production

Propagules are the infective agent of fungal BCAs. Mass-scale production of commercial BCAs has been successfully achieved both from submerged fermentation (SmF) and solid-state fermentation (SSF) approaches, with different product characteristics depending on the used system (Pham et al., 2010., Mascarin and Jaronski, 2016). The growth of filamentous fungi occurs naturally on culture media surfaces. In unstirred liquid media, a layer of mycelium appears with spores, whereas mycelia or pellets are produced with agitation, making spore formation highly difficult and causing dispersion. In solid cultures, mycelium growth is carried out on the solid surface, but also in the cavities with free spaces, homogeneously colonizing the media. Spore yields decrease with excess water since the cavities are occupied by water and are thus unavailable for fungal growth (De la Cruz Quiroz et al., 2015). Process optimization focuses on improving both conidia production and quality in terms of activity conservation. Both are essential for the supply of biopesticides based on entomopathogenic fungi, which in turn are affected by many other variables (Muñiz-Paredes et al., 2017).

Three types of propagules can be produced by entomopathogenic fungi, including spores, mycelium, and chlamydospores, each of them possessing their own characteristics in terms of production, stability and biocontrol activity. Most of the produced BCAs use spores as active ingredient due to difficulties in downstream processing of mycelia and chlamydospores, even though mycelia biocontrol activity is also excellent and chlamydospores present better stability than mycelia (Verma et al., 2007). Regarding spore production, aerial spores (namely conidia) produced by SSF and submerged spores (namely blastospores or submerged conidia) produced by SmF should be distinguished. The differences between aerial conidia and blastospores/submerged conidia are shown in Table 1.1. The morphological, functional, and biochemical differences displayed by aerial conidia in contrast to blastopores and submerged conidia enable them to persist longer in harsh environmental conditions (Hölker et al., 2004; Lopez-Perez et al., 2015), effectively promoting aerial conidia as the preferred propagules for fungal biopesticide production. Differences in thermotolerance between conidia in different entomopathogenic fungal species have also been presented (Souza et al., 2014). Most BCAs are produced by species

that present conidia with a hydrophobic surface, which are more thermotolerant than the hydrophilic ones, and thus are more prone to resist environmental conditions when used as biopesticides.

**Table 1.1.** Main differences between propagules produced by fungal entomopathogens.

| | **Aerial conidia** | **Blastospores/ submerged conidia** | **Reference** |
|---|---|---|---|
| **Production mode** | SSF | SmF | Mascarin and Jaronski, (2016) |
| **Infectivity** | Insects living in low humidity environment | Insects living in the high humidity boundary of the phylloplane | |
| **Germination** | Slower infection and germination | Faster infection and germination | |
| **Propagule-host environment** | Generally shared | Generally not shared | Schrank and Vainstein, (2010) |
| **Viability** | Longer | Shorter | Muñoz et al. (1995) |
| **Morphology** | Thinner, thicker outer wall | Larger, thinner outer walls, not clustered | |
| **Uv-resistance** | Higher resistance | Lower resistance | |
| **Cytoplasm** | Few organelles | Many cytoplasmic organelles | |
| **Bundles of fascicles** | Presence | Absence | Holder et al. (2007) |
| **Virulence** | Higher virulence | Lower virulence | |
| **Resistance** | Higher resistance to abiotic factors | Lower resistance to abiotic factors | Faria and Wraight, (2007) Muñiz-Paredes et al. (2017) |
| **Hydrophobicity** | Highly hydrophobic nature. Binding to hydrophilic surfaces | Hydrophilic nature, particularly its surface. Binding to hydrophobic surfaces. Properties sometimes between hydrophilic and hydrophobic | Muñoz et al. (1995) Holder et al. (2007) |

SSF is the most used method to produce fungal infective propagules (De la Cruz Quiroz et al., 2015; Mascarin and Jaronski, 2016). As opposed to SmF, SSF is defined as a process that occurs in the absence or near absence of free water, generally using a natural substrate as a carbon and energy source. The substrate must possess enough moisture to support both growth and metabolic activity of the microorganism (Thomas et al., 2013). The intrinsically low moisture levels typical of solid cultures represent the main difference between SSF and SmF (Manpreet et al., 2005). The most relevant advantages and disadvantages of SSF in comparison with SmF according to different authors (Pandey, 2003; Botella et al., 2005; Couto and Sanromán, 2006; Hölker et al., 2004; Thomas et al., 2013) are presented in Table 1.2.

**Table 1.2.** Advantages and disadvantages of SSF in comparison to SmF.

| SSF advantages | SSF disadvantages |
|---|---|
| • Reduced costs due to low water needs, implying low residual water treatment.<br>• Better yields and lower capital investment due to the generalized use of agro-industrial residues as substrates.<br>• Lack of foams.<br>• Softer or null agitation.<br>• Better aeration due to the porosity given by the bulking agent, ensuring high oxygen transfer to the microorganisms. | • Difficulties on homogenizing the medium, causing temperature and composition gradients, among others.<br>• Metabolic heat exchange usually presents an important problem, especially when working at large scale, complicating temperature control.<br>• Scale-up difficulties.<br>• Substrate nature difficults measurements and monitoring of operational parameters (pH, temperature, moisture, substrate and product concentrations and nutrient conditions).<br>• Fermentation time is higher due to the general use of microorganisms with low specific growth rate. |

### 1.3.2. Variety of substrates used for fungal SSF

Substrate selection is one of the most important steps to go through when starting a fungal SSF process. A wide variety of substrates used for SSF fungal conidia production are shown in Table 1.3. Substrate selection depends on several factors, mainly cost and availability. All solid substrates have features in common, primarily their macromolecular structure, which is normally composed of cellulose, lignocellulose, pectin or other polysaccharides. The natural material serves both as nutrient source and as support for

fungal growth. However, solid media is degraded during fungal growth, causing changes in its physical and geometrical features which might cause changes in heat and mass transfer (Pandey et al., 2008; De la Cruz Quiroz et al., 2015).

**Table 1.3.** Substrates used for fungal conidia production with various entomopathogenic fungi.

| Fungal strain | Solid substrate/support | Reference |
|---|---|---|
| *Beauveria bassiana* | Refused potatoes and sugar-cane bagasse (60%-40%) | Santa et al. (2005) |
| | Rice husk, wheat bran, pigeon pea husk, urad husk, pongamia seed cake, jatropha seed cake and tea leaf waste | Mishra et al. (2016) |
| | Wheat bran and rice bran | Dhar et al. (2016) |
| | Rice, crushed sorghum, wheat bran and rice bran | Dhar (2011) |
| | Parboiled rice | Tarocco et al. (2005) |
| | Rice and crushed sorghum | Dhar (2011) |
| | Wheat bran | Nuñez-Gaona et al. (2010) |
| | Rice | Ye et al. (2006) |
| | | Xie et al. (2012) |
| | White rice | Pham et al. (2010) |
| *Beauveria*<br>*Metarhizium*<br>*Isaria*<br>*Paecilomices* | Rice, wheat, rye, corn and sorghum | Mar and Lumyong (2012) |
| *Trichoderma harzianum* | Straw and wheat bran (33.3%-66.6%) | Zhang and Yang (2015) |
| | Different combinations of wheat straw, wheat bran and wheat grain | Mishra and Sundari (2017) |
| *Trichoderma asperellum* | Polyurethane foam and rice husk | Barrera et al. (2019) |
| *Trichoderma ghuizhouense* | Stevia residue, rice bran, wheat bran, rice chaff, rice straw | Hong-jun et al. (2021) |
| Various *Trichoderma* strains | Wheat bran and corn | Cavalcante et al. (2008) |

**Table 1.3. (cont).** Substrates used for fungal conidia production with various entomopathogenic fungi.

| Fungal strain | Solid substrate/support | Reference |
|---|---|---|
| *Trichoderma*<br>*Aspergillus*<br>*Rhizopus* | Grape wine residue | Jin et al. (2016) |
| *Trichoderma viride* and *Aspergillus niger* | Pineapple waste | Omwango et al. (2013) |
| *Metarhizium anisopliae* | Rice | Bhanu Prakash et al. (2008) |
| | Broken maize | Moslim et al. (2005) |
| | Rice and sugarcane bagasse (9:1) | Da Cunha et al. (2020) |
| *Metarhizium robertsii* | First quality rice grains | Méndez-González et al. (2020) |
| *Clonostachys rosea* | Wheat bran and maize meal | Zhang et al. (2014) |
| *Conithyrium minitans* | Wheat bran | Liu et al. (2018) |

Several studies have focused on substrate optimisation, most of them using BB as BCA. In many of these works, the optimal chosen substrate turns out being a combination of various substrates, particularly when using agro-industrial wastes due to their synergistic effects. Most solid substrates must undergo some sort of pre-treatment prior to the SSF process (Raimbault, 1998; De la Cruz Quiroz et al. 2015).

Typically, fungal SSF substrates can be divided into two main categories: crops and agro-industrial wastes. Crops were the first used substrates to perform fungal SSF bioprocesses, and among them rice has been recognised by many authors as an optimal substrate (Jenkins et al., (1998); Posada-Flórez, (2008); Lopez-Perez et al., (2015) and Muñiz-Paredes et al., (2017)). Other crops such as cereal gains are also commonly used for mass production of aerial conidia, as stated by Moslim et al. (2005) and Jaronski (2014). However, using crops as substrates presents major drawbacks, as they represent a reduction in the quantity of available food in a world in which food scarcity has arisen as an enormous problem. This major issue is overcome with the use of agro-industrial residues as SSF substrates, which can provide sufficient nutrients for fungal growth while also serving as support (De la Cruz Quiroz et al., 2015). It must be pointed out that by

promoting the use of waste and waste mixtures as substrates, the obtained products present an added value due to residue valorisation, a mostly relevant assumption in the actual frame of a circular economy (Ballardo et al. 2017).

Agro-industrial wastes have gained importance during recent years for conidia sporulation due to a considerable reduction in production cost (Lopez-Perez et al. 2015), although their real potential as substrates is yet to be harnessed (Balasubramanian and Tyagi, 2017). As shown in Table 1.3, a wide variety of residual wastes has been used, most of them being low-cost residues or poorly used by-products, so their use contributes to reduce environmental pollution. Nevertheless, physical and chemical characterization of agro-industrial wastes is sometimes complex due to their heterogeneity. Nutrient content and availability in grains and forages have different sources of variation. Plant variety and geographical origins are considered the most relevant, causing difficulties in maintaining consistency between batches (Lopez-Perez et al. 2015).

Among agro-industrial wastes, a relevant volume is composed of cellulosic materials, which might be better suited for SSF due to their high water absorption capacity in comparison to that of starchy materials (Santa et al. 2005). Many fungal strains are able to hydrolyse cellulose and lignocellulose using enzymes (Novy et al. 2015; Sánchez, 2009). De la Cruz Quiroz et al. (2015) stated that starchy and lignocellulose materials are the most used substrates in fungal SSF processes. Nevertheless, lignocellulosic wastes are mostly used for enzyme and bioethanol production rather than for germinating conidia (Kelbert et al. 2015; Cheirsilp and Kitcha, 2015).

### 1.3.3. Fungal SSF reactor design

Typical SSF reactor designs can be found in Figures 1.6. and 1.7. (Krishania et al., 2018; Méndez-González et al., 2018; Méndez-González et al, 2017; Xie et al, 2012; Pham et al. 2010). Comparison between different types of reactors is always complicated since achieving similar conditions is difficult, resulting in conflicting experimental data. This highlights the noteworthy relevance of reactor design and engineering in entomopathogenic fungi SSF, even though comparisons of conidial yields between different reactor designs are very scarce (Muñiz-Paredes et al. 2017). Reactors used in different works (when described) are listed in Table 1.4.

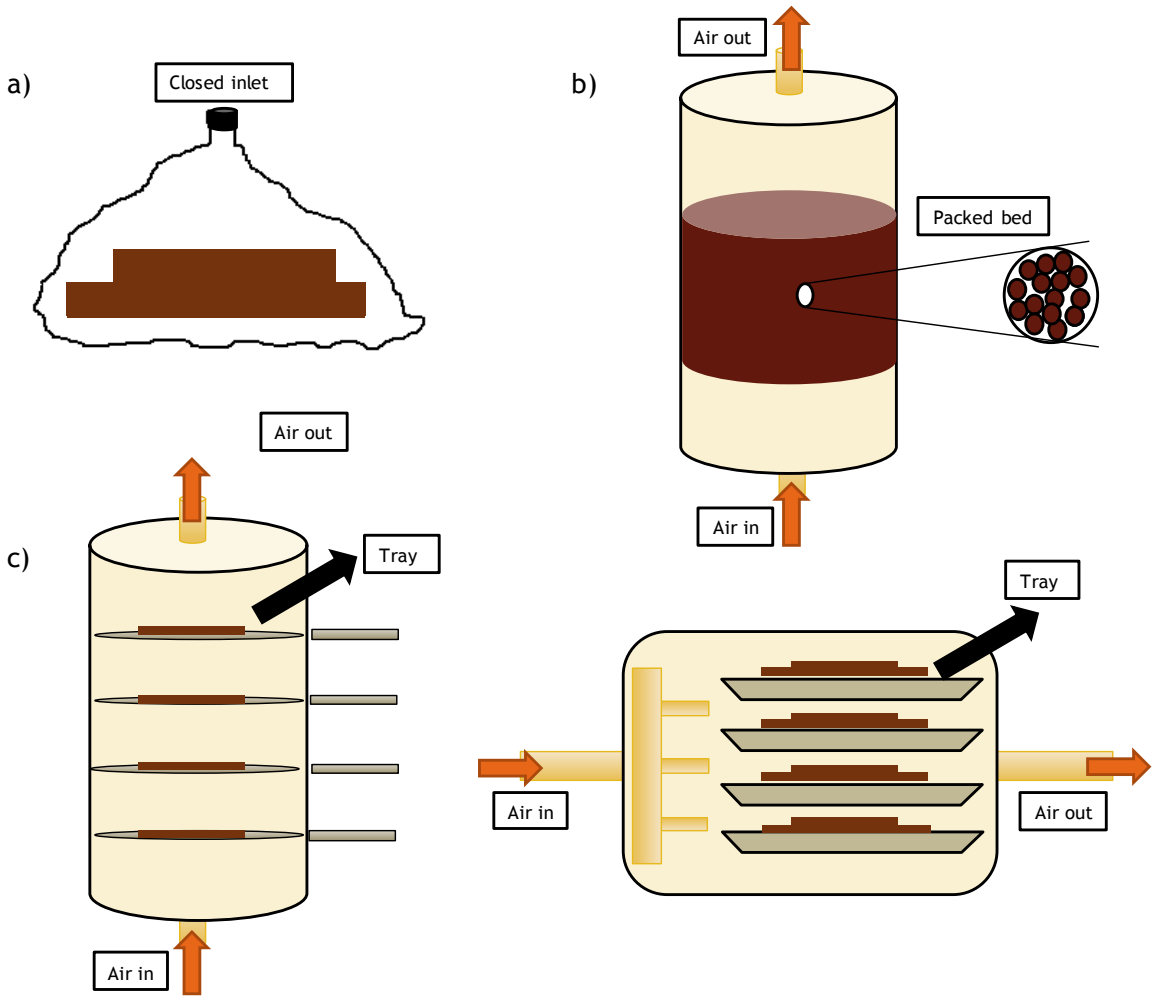



**Figure 1.6.** Schematic representation of typical reactor designs in fungal SSF: a) polypropylene bags, b) packed bed reactor and c) tray reactors.

Polypropylene bags have traditionally been preferred for commercial-scale production (Couto and Sanromán, 2006). However, tray bioreactors are currently used in most industrial processes (Nigam and Pandey, 2009., Krishania et al., 2018., Mascarin et al., 2019), even though they require far more space than their predecessors. Their simplicity, low cost, ease of operation and superior parameter control have made them preferable over polypropylene bags (Thomas et al. 2013, Krishania et al., 2018). Bed thickness is a crucial limitation for increasing productivity in tray bioreactors, as acknowledged by Xie et al. (2012) (increasing substrate thickness yielded worst conidia production). Relevant differences in substrate bed thickness have been reported by some authors. Jou and Lo (2011) reported an optimal bed thickness for fungal growth and activity in trays at 28°C and 95% relative humidity of 1.0 cm, which maintained constant air temperature in static tray bioreactor. Krishania et al. (2018) pointed that substrate thickness might be of 5 to 15 cm per tray.

The type of reactor used determines aeration conditions. Traditional polypropylene bags and flasks are systems with no forced aeration, resulting in limited gas exchange and limited heat transfer, and are solely used if natural gaseous exchange with the environment is sufficient (Lopez-Perez et al. 2015). In comparison, both column and tray bioreactors air inlets allow gaseous exchange both for $O_2$ and $CO_2$, as well as heat and volatile compound removal. Column reactors might be also presented as rotatory drums, providing better aeration and limited damage to the inoculum or product at the cost of only using 30% reactor volume as operating volume (Couto and Sanromán, 2006). In both trays and columns, mass and heat transfer limitations present a serious drawback when scaling, causing gradients within the substrate bed and diminishing total productivity (Pandey et al., 2008; Krishania et al., 2018).

Many works have used different reactor configurations at lab scale, even though the best results from scaling up have still been achieved using the tray configuration (Thomas et al. 2013). However, the development of packed bed bioreactors (PBBs) has gained relevance in the last years since they are much easy to handle and less labour intensive in comparison to trays (Krishania et al., 2018). Méndez-González et al. (2020) compared the three configurations using *Metarhizium robertsii* at lab scale, obtaining highest conidia production and productivity with PBB. Despite its advantages, recent efforts and achievements, to the author's knowledge, no industrial conidia production has been successful with PBBs, which highlights the need of more research on this reactor design to use them to produce fungal conidia by SSF (Méndez-González et al., 2018, da Cunha et al., 2020).

### 1.3.4. Culture conditions in fungal SSF

To properly run a SSF process, it is necessary to consider a wide range of parameters, which are summarized in Figure 1.7. However, scarcity of information relating to the optimization of several parameters is still common, which highlights the need to research on them for every fungal SSF fermentation. The final aim in any optimization process should be obtaining the maximum conidial yield while ensuring high conidial virulence (Garza-López et al. 2012; Rodríguez-Gómez et al. 2009). Also, ensuring the lowest cost, particularly during downstream processing, requires the use of post-harvesting strategies such as formulation and drying processes (Mascarin and Jaronski, 2016).

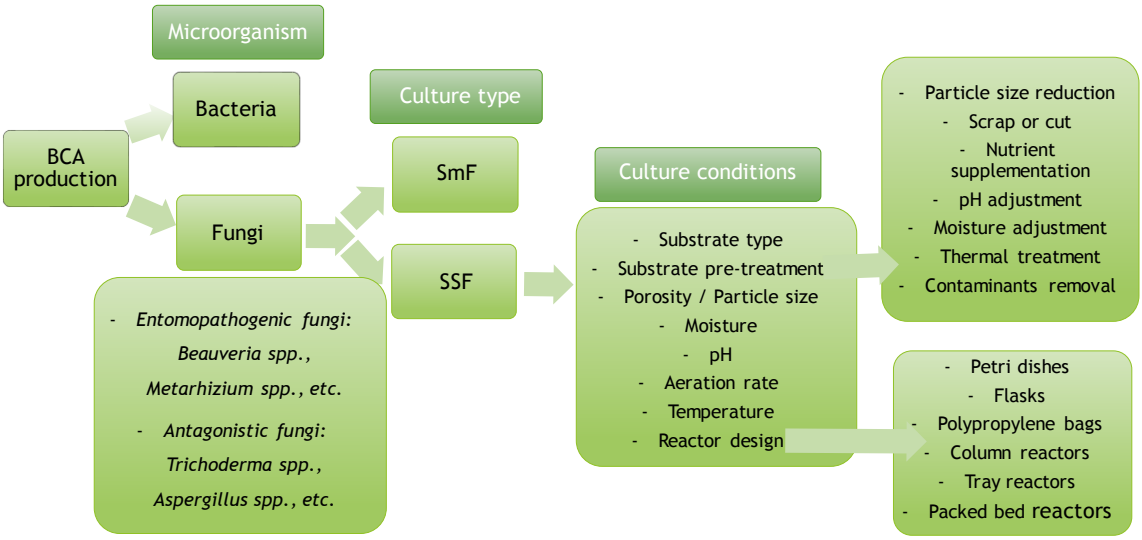



**Figure 1.7.** BCA production summary: microorganisms, reactors and culture conditions.

After an extensive literature review which is summarized in Table 1.4, parameters affecting conidia SSF production have been classified in two groups depending on its relevance on the production process: major parameters (temperature, moisture, aeration rate, inoculum size/concentration and porosity/particle size) and minor parameters (pH, C/N ratio, nutrient supplementation and inoculum age) (Krishna, 2005; Hallsworth and Magan, 1996; Pandey et al., 2008, De la Cruz Quiroz et al., 2015; Muñiz-Paredes et al., 2017; Méndez-González et al., 2020).

**Table 1.4.** Summary of SSF conidia production with its main parameters.

| Fungal strain | Area ($cm^2$) or volume (mL) | Type of reactor | Substrate moisture (%) | Aeration rate (mL $min^{-1}$) or $\%O_2$ | Temp (ºC) | pH | Inoculum size/ concentration (% v:v and/or spores $mL^{-1}$) | C/N ratio | Nutrient addition | Conidia production (conidia $g^{-1}dm$) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Beauveria bassiana* | 2400 $cm^2$ per tray | Tray bioreactor | 40 | (-) | 25 | (-) | 10% | (-) | No | Max achieved $3.94x10^9$ | Xie et al. (2013) |
| | 63.61 $cm^2$ | Petri dishes | 50 | No | 25 | (-) | 10% | No | Yes | $6.23x10^9$ (d) | Yang et al. (2010) (a) |
| | 500 mL | Erlenmeyer flasks | 40 – 67 | No | 28 | (-) | $1x10^8$ | 2.5 – 22.67 | Yes | $7.6x10^3$ – $1.8x10^9$ | Mishra et al. (2016) |
| | 75 mL | Serological bottles | 40 | 16% (b) | 28 | (-) | $1x10^6$ | (-) | No | $1.51x10^9$ (d) | Garza-López et al. (2012) |
| | 75 mL | Serological bottles | 66 (b) | No | 28 | (-) | $5x10^7$ (b) (e) | (-) | No (b) | $1.18x10^{10}$ | Núñez-Gaona et al. (2010) |
| | 250 mL | Erlenmeyer flasks | 75 (b) | No | 26 | 6.0 | $1x10^7$ | (-) | No | $3.5x10^9$ (d) | Santa et al. (2005) |
| | 1000 mL | Column bioreactor | 65 (b) | 60 (b) | | | | | | $9.9x10^9$ (d) | |

**Table 1.4. (cont)** Summary of SSF conidia production with its main parameters.

| Fungal strain | Area ($cm^2$) or volume (mL or L) | Type of reactor | Substrate moisture (%) | Aeration rate (mL $min^{-1}$) or $\%O_2$ | Temp (ºC) | pH | Inoculum size/ concentration (% v:v and/or spores $mL^{-1}$) | C/N ratio | Nutrient addition | Conidia production (conidia $g^{-1}$dm) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Beauveria bassiana* | Not stated | Petri dishes | (-) | Not stated | 25 (b) | 7.0 (b) | $1x10^5$ | 5:1 | Yes (b) | $2.66x10^7$ (d) | Dhar et al. (2016) |
| | 250 mL | Flasks | 20-50 (b) | No | 26 | 6.0 (b) | (-) | (-) | No | $2.6x10^9$ (d) | Tarocco et al. (2005) (a) |
| | Not stated (20 $m^2$ room) | Tray bioreactor | 38 | No | 25 | (-) | (-) | (-) | No | $1.7\ x10^{11}$ (u) | Ye et al. (2006) |
| | Not stated | Non specified container | 40 (b) | No | 25 (b) | (-) | 10%, $1x10^7$ (b) | (-) | No | 0.0405 (c) | Pham et al. (2010) |
| | 1300 mL | Biofilm | (-) | 1500-2500 | 30 | 6.0 | (-) | (-) | Yes | $1.23x10^9$ (x) | Lara-Juache et al. (2021) |
| *Trichoderma harzianum* | Not stated | Bottles | 75 (b) | No | 30 (b) | 6.0 (b) | $1x10^7$ (b) (i) | (-) | Nitrogen (b) | $1.49x10^{10}$ (d) (h) | Zhang and Yang, (2015) |
| *Trichoderma asperellum* | 16 L total Tray area not stated | Tray bioreactor/ fixed bed | 97.5 or 76.9 (for each substrate) | 420-1130 (b) | 23-28 (b) | (-) | $1x10^5$-$1x10^6$ (b) | 18.1 (b) | Yes | $2.57x10^7$ (d) (q) | Barrera et al. (2019) |

**Table 1.4. (cont)** Summary of SSF conidia production with its main parameters.

| Fungal strain | Area ($cm^2$) or volume (mL) | Type of reactor | Substrate moisture (%) | Aeration rate (mL $min^{-1}$) or $\%O_2$ | Temp (ºC) | pH | Inoculum size/ concentration (% v:v and/or spores $mL^{-1}$) | C/N ratio | Nutrient addition | Conidia production (conidia $g^{-1}dm$) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Trichoderma harzianum / viride* | 250 mL | Erlenmeyer flasks | 33-73 | No | 30 | (-) | $1x10^6$ | (-) | No | $28.30x10^8$ (d) (TH*)* $24.10x10^8$ (d) *(T.viride)* | Cavalcante et al. (2008) |
| *Trichoderma virens* | 2924 $cm^2$ per tray (2-3) | Tray bioreactor | 50 | 2.5 (k) | 28 | (-) | (-) | (-) | No | $1x10^{10}$ (h) | Jou and Lo, (2011) |
| *Trichoderma viride Aspergillus niger* | 500 mL | Beakers | (-) | (-) | (-) | (-) | 2% | (-) | No | Achieved but not stated | Omwango et al. (2013) |
| *Trichoderma pseudokoningii* | Not stated | Sterilised bags | 50 | No | 25 | (-) | 20 (g) | (-) | Yes | Not stated | Chu et al. (2017) |
| *Trichoderma atroviride* | 63.61 $cm^2$ | Petri dishes | No | No | 25 (b) | (-) | $2x10^5$ | (-) | No | $1.51x10^9$ (d) (l) | Daryaei et al. (2016) |
| *Trichoderma guizhouense* | Not stated | Not stated | 50-70 | No | 28 | 3.0 | $1x10^8$ (v) | (-) | Amino acids | $7x10^9$ (d) (w) | Hong-jun et al. (2021) |

**Table 1.4. (cont).** Summary of SSF conidia production with its main parameters.

| Fungal strain | Area ($cm^2$) or volume (mL) | Type of reactor | Substrate moisture (%) | Aeration rate (mL $min^{-1}$) or $\%O_2$ | Temp (ºC) | pH | Inoculum size/ concentration (% v:v and/or spores $mL^{-1}$) | C/N ratio | Nutrient addition | Conidia production (conidia $g^{-1}dm$) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Trichoderma Aspergillus Rhizopus* | 250 mL | Erlenmeyer flasks | 65 | No | 30 | (-) | $1x10^7$ | (-) | Yes [b] | Achieved but not stated | Jin et al. (2016) |
| *Metarhizium anisopliae* | Not stated | Polypropylene bags | 22.3 - 75.68 [b] | No | 26 | 6.76. 0-7.1 [b] | $1x10^6$ | (-) | Yeast extract [b] | $5.3x10^{10}$ $4.7x10^{10}$ $4.5x10^{10}$ [d] | Bhanu Prakash et al. (2008) [j] |
| | Not stated | Column bioreactors | 57-58 [b] | 0 - 180 [b] | 25 | (-) | $1x10^7$ | (-) | (-) | $7x10^9$ [d] | Arzumanov et al. (2005) |
| | Not stated | Column bioreactors | 47 | 0.34 [n] | 27 | (-) | $1x10^6$ | (-) | No | $2.8x10^{11}$ [o] | Dorta and Arcas, (1998) |
| | Not stated | Polypropylene bags | (-) | No | 28 | (-) | Not stated | (-) | No | 0.042 [c] | Moslim et al. (2005) [e] |
| | 6 units of 100 $cm^2$ or 400 $cm^2$ each | Column bioreactor, packed bed | 48 | 1000 or 12000 | 28.2 – 29.8 [t] | (-) | $1x10^7$ [c] | (-) | No | $4.02x10^9$ Max average achieved | da Cunha et al. (2020) |

**Table 1.4. (cont).** Summary of SSF conidia production with its main parameters.

| Fungal strain | Area ($cm^2$) or volume (mL) | Type of reactor | Substrate moisture (%) | Aeration rate (mL $min^{-1}$) or $\%O_2$ | Temp (ºC) | pH | Inoculum size/ concentration (% v:v and/or spores $mL^{-1}$) | C/N ratio | Nutrient addition | Conidia production (conidia $g^{-1}$dm) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Metarhizium flavoviride* | 63.61 $cm^2$ | Petri dishes | (-) | No | 24 (b) | (-) | $6x10^6$ | No | No | $1x10^5$ (m) | Thomas and Jenkins, (1997) |
| *Metarhizium flavoviride* | Not stated (1kg per bag) | Polypropylene bags | 35 – 60% | Passive aeration | 25 | (-) | Not stated (at least $6x10^6$) | (-) | No | $1.5x10^9$ $4\text{-}5x10^{10}$ (f) | Jenkins et al. (1998) (e) |
| *Metarhizium robertsii* | 30x40 cm bags 70x30x3cm trays (x12) 20x2 cm columns (number not stated) | Polypropylene bags Tray bioreactor Column bioreactor (b) | 30% | Variable, 0.1 to 1 in tray, 0.16 to 1.28 in columns (L $Kg_{wm}^{-1}$ $min^{-1}$) (b) | 26 to 32 (b) | (-) | $2x10^6$ (p) | (-) | No | $1.58x10^9$ (d) | Méndez-González et al. (2020) |
| *Clonostachys rosea* | 80000 $cm^2$ per tray | Tray bioreactor | Between 50 and 70% | (-) | 24 | (-) | $7.5x10^7$ (e) | (-) | No | $3.36x10^{10}$ (d) | Zhang et al. (2014) |
| *Conithyrium minitans* | 40 L tank volume, 250 mL per flask | Special tray bioreactor | (-) | GDD (r) | 20 to 28 | 7.0 | (-) | (-) | Yes | $1.5x10^{10}$ (d) | Liu et al. (2018) |

**Table 1.4. (cont).** Summary of SSF conidia production with its main parameters.

| Fungal strain | Area ($cm^2$) or volume (mL) | Type of reactor | Substrate moisture (%) | Aeration rate (mL $min^{-1}$) or $\%O_2$ | Temp (ºC) | pH | Inoculum size/ concentration (% v:v and/or spores $mL^{-1}$) | C/N ratio | Nutrient addition | Conidia production (conidia $g^{-1}dm$) | Reference |
|---|---|---|---|---|---|---|---|---|---|---|---|
| *Metarhizium anisopliae* *Beauveria bassiana* | 63.61 $cm^2$ | Petri dishes | 5 | No | 25 | (-) | $1x10^7$ | No | No | $10x10^{11}$ (d) | Kassa et al. (2008) |
| | 63.61 $cm^2$ | Petri dishes | (-) | (-) | (-) | (-) | $1x10^6$ | 10:1 to 75:1 (b) | Yes (b) | $3.75x10^7$ (d) | Safavi et al. (2007) |
| | Not stated | Petri dishes | (-) | No | 25.8-28.2 | 6.5 | (-) | (-) | Yes (b) | $2.05x10^{10}$ (d) (s) | Balakrishnan et al. (2011) |
| *Isaria* *Beauveria* *Metarhizium* *Penicillium* | Not stated (10 cm height) | Test tubes | Stable, value not stated | (-) | 25 | (-) | (-) | (-) | No | $530.6x10^9$ (d) | Mar and Lumyong, (2012) |

(a): statistic modelling study; (b): optimized parameter; (c): g conidia $g^{-1}dm$; (d): best production obtained, typically in tests where various substrates are tested, or best production for each tested substrate; (e): mass production process; (f): conidia $g^{-1}$ dry powder (final product); (g): mL inoculum $kg^{-1}$substrate; (h): CFU $g^{-1}$; (i): CFU $bottle^{-1}$ (volume not stated); (j): response surface methodology (RSM) study; (k): m $s^{-1}$; (l): conidia $mL^{-1}$; (m): conidia $insect^{-1}$; (n): L $h^{-1}$ $g^{-1}$ initial dm; (o): spores $L^{-1}$; (p): conidia $g^{-1}dm$; (q): productivity: conidia $g^{-1}dm$ $h^{-1}$; (r): GDD: gas double-dynamic; (s): conidia $100mL^{-1}$; (t): average temperature; (u): conidia $g^{-1}$ dry powder; (v): CFU $mL^{-1}$; (w): CFU $g^{-1}dm$; (x): spores per gram of support.

#### 1.3.4.1. Major parameters

**Aeration rate:** since they are aerobic organisms, fungi must be provided with some sort of aeration to maximize both growth and sporulation. As stated in section 1.3.3, aeration can only be optimised in column or tray configuration. Majorly, authors agree on the relevance of aeration in fungal SSF growth for various reasons: it supplies oxygen but also removes $CO_2$ and excess heat, while displacing other volatile metabolites that might cause problems if they accumulate (Krishna, 2005) and enhances spore production (Santa et al., 2005, Méndez-González et al., 2020) specially in column bioreactors. Regarding heat removal, even though most of the produced metabolic heat can be transferred to the air as latent heat or water vaporization, optimal water content has to be maintained in order to make up for water loss during fermentation (Dorta and Arcas, 1998). Lopez-Perez et al. (2015) stated that BB is sensitive to a 5% increase in $CO_2$ concentration, whereas Papavizas (1985) stated that *Trichoderma* spp. can withstand up to 10%. Méndez-González et al. (2020) obtained maximum conidia production when $CO_2$ production rate dropped by 50%. $CO_2$ studies have also demonstrated that fungal vegetative growth is not always proportional to sexual one, as presented by da Cunha et al. (2020) performing successive cultivations of *Metarhizium anisopliae*. However, different aeration rates do not always affect conidia production, as found by Arzumanov et al. (2005) with *Metarhizium anisopliae*.

It is noteworthy to mention that, while considering the optimal aeration rate for an SSF system, parameters such as temperature, heat removal, and moisture content should also be considered, given the close relationship between them.

**Temperature:** optimal temperature highly depends on the fungal strain, even though it can vary as a function of the fungal infection cycle stage (Hallsworth and Magan, 1996). From the reviewed literature, temperatures in the range of 25–30°C are the most common among the studied strains (Table 1.4).

Generally, *Beauveria*'s upper temperature growth limit is of 34–36°C Higher temperatures may greatly reduce efficacy (Ugine, 2011; Mascarin and Jaronski, 2016). Using BB, Santa et al. (2005) pointed out that temperature was not as relevant for conidia production as other parameters, with its optimal value always located at 25 ± 1°C (Yang et al. 2010; Xie et al. 2012; Pham et al. 2010). Regarding *Trichoderma* strains, existing literature reveals a wide range of temperatures in which the species can grow, varying from 0°C in *Trichoderma polysporum* to 40°C in *Trichoderma koningii* (Tronsmo and

Dennis, 1978), even though the optimal growth temperature for most *Trichoderma* spp. strains range is between 25–30°C (Kubicek and Harman, 1998). Daryaei et al. (2016) found that the temperature at which conidia are produced affects both germination and bioactivity, implying that formulations consisting of conidia obtained in the highest germination yield might not result in optimal bioactivity, requiring a trade-off between quantity and quality.

Despite being one of the most well-known fungal growth SSF parameters, temperature control becomes more challenging when scaling up. Consequently, it is a key factor when designing reactors at bench or industrial scale. Moreover, it is highly related with forced aeration, mostly due to metabolic heat removal-associated difficulties (Krishna, 2005., Pandey et al., 2008).

**Moisture:** regardless of the chosen substrate, moisture is one of the critical parameters to consider while optimizing fungal SSF processes. Substrates need to possess a minimal water content to ensure fungal growth and metabolism. As presented in Table 1.4, optimal values typically vary between 40-80% (w/w). Same organisms can grow in different substrates differing in water holding capacity, thus making it impossible to rely on the amount of moisture alone regardless of the nature of the substrate (Manpreet et al. 2005). An adjustment is sometimes needed to achieve sufficient initial moisture values for fungal growth.

Moisture content primarily depends on the chosen substrate, as well as on the process constitution, as shown by Mishra et al. (2016) using seven different substrates with BB. Some substrates, mainly crops, have been optimized for specific fungi (data shown in Table 1.4). Working with various *Trichoderma* strains, Cavalcante et al. (2008) stated that the use of low-moisture substrates inhibits fungal growth. However, surpassing certain values also results in losses in spore production, with optimal values ranging from 40–65% depending on the substrate, values used recently by others authors (Chu et al. 2017). Using response surface methodology, Bhanu Prakash et al. (2008) inferred optimal moisture content to optimize conidial yields of *Metarhizium anisopliae* as 75.68% for sorghum, 73.21% for barley, and 22.34% for rice. Using the same substrate and fungal strain, Santa et al. (2005) found optimal moisture values of 75% when working in Erlenmeyer flasks (250 mL), while this value was lowered to 65% when working in a 1000 mL column type reactor with forced aeration. Lower initial moisture was needed while providing aeration with saturated air, which might have helped maintain substrate moisture. Moisture evolution throughout the fermentation must also be taken into

consideration, particularly when scaling, as moisture loss might result in a decrease in spore productivity. In a 16 L total volume reactor working with rice husk and polyurethane foam, Barrera et al. (2019) suffered moisture loses from initial values between 76.9-97.5% to final values of 32.1%.

Moisture arises as a relevant optimization parameter in fungal SSF processes with values mainly depending on the substrate, on the selected fungal strain, scale of the process and reactor configuration.

**Inoculum size/concentration:** as shown in Table 1.4, inoculum concentration values are often presented in SSF fermentation studies. Some studies have focused on determining the optimal inoculum concentration. For BB, some reports indicate that the optimum concentration lies between $1\times10^6$ and $1\times10^7$ conidia per gram of dry matter ($g^{-1}$dm) (Nuñez-Gaona et al. 2010; Pham et al. 2010; Santa et al. 2005), for *Trichoderma asperellum* the optimum concentration was of $1x10^7$ conidia per $g^{-1}$dm (Barrera et al., 2019) and for *Trichoderma viride,* concentrations of $1\times10^7$ CFU $bottle^{-1}$ (volume not stated) were reached. Lower values were not enough to produce conidiation and higher values were discarded due to the toxins produced from conidia accumulation (Zhang and Yang, 2015).

Nevertheless, it must be taken into consideration that these results could vary if using non-sterilized residues, hereby affecting inoculum concentration.

**Porosity/particle size:** particle size reduction is nearly mandatory when using agro-industrial wastes as substrates (De la Cruz Quiroz et al. 2015). With sugarcane bagasse, Membrillo et al. (2011) reported that the size and geometry of the substrate particles affect fungal specific growth rate, as well as product yields. Manpreet et al. (2005) identified the optimal substrate particle size as between 1 mm and 1 cm; this parameter represents a compromise between the accessibility of the nutrients and the availability of oxygen. Santa et al. (2005) found an optimal particle size range between 0.8–2 mm using refused potatoes as substrate for BB sporulation in 250-mL Erlenmeyer flasks. Pandey (1992) reported higher productivities while working in 500 mL erlenmeyers with substrates containing mixed particle sizes between 180 μm to 1.4 mm. Moreover, it is important to remember that particle size tends to diminish during SSF processes (Krishna, 2005).

Porosity depends not only on the substrate but also on the reactor scale. Ideally, small particle size could provide a larger surface area for microbial attachment, even

though an extremely small particle size would cause substrate agglomeration, affecting oxygen transfer and retarding microorganism development. On the other hand, large particle sizes provide better oxygen transfer but limit surface area for fungal attachment (Yazid et al. 2017).

Despite its influence in SSF process optimization, accurate particle size values do not exist due to the heterogeneity of the processes, so an optimal value range must be selected for each particular process depending on the studied substrates (Pandey et al., 1999).

#### 1.3.4.2. Other parameters

**Mixing/agitation:** mixing/agitation have an important influence in SSF processes, as they help at having correct oxygen transfer throughout the substrate as well as helping at heat removal. Despite its usefulness mixing is not optimized in most cases, as it is not advisable depending on the characteristics of the used substrate, type of reactor or fungal strain. It is specially complicated to apply agitation properly when working with fungal strains, as it might disrupt mycelial attachment to the solid support, thus affecting spore production (Krishna, 2005). Most fungal optimization studies opt for not applying agitation, however, some studies like Zhang et al. (2014) have successfully applied mixing techniques (in this case growing *Clonostachys rosea* in wheat bran and maize meal (3:1)), demonstrating that mixing might be favourable for fungal sporulation when applied correctly.

**Inoculum age:** despite being though to play an important role in the fermentation process (as conidia quality tends to diminish with age), this factor is still not often studied in conidia production, according to Muñiz-Paredes et al. (2017). So far, obtained results are scarce, sometimes controversial and strongly strain dependant. In several Metarhizium spp. strains, *Isaria* and BB, conidia germination tends to decrease as the culture ages (Smith and Edgington, 2011; Hallsworth and Magan, 1996). Nevertheless, in some in some *Isaria fumosorosea* strains and in *Metarhizium anisopliae* viability increases as culture ages, probably due to maturation (Muñiz-Paredes et al. 2016; Moslim et al. 2005).

**pH:** as shown in Table 1.4, pH is often ignored during optimisation procedures, mainly due to it being more dependent on the selected substrate than on the strain. Correct pH regulation is a complex process when working on SSF, as it tends to vary mainly due

to changes caused by growth characteristics and also because of the nitrogen source (Krishna, 2005). Conidial fungi can grow over a wide range of pH values; most fungal strains tolerate a pH range from 4 to 9 but grow and sporulate maximally near neutral pH (Papagianni, 2004). Some studies have highlighted major relevance regarding pH effect in fungal SSF, effectively showing significant interactions between various parameters. Mishra et al. (2016) stated that an elevation in the pH of the system caused by hydroxide ion accumulation might limit growth in BB. Santa et al. (2005) reported pH as one of the most influencing parameters in BB conidia production and Zhang and Yang (2015) reported an optimal pH value of 6.0 for conidia production in TH. Some *Trichoderma* strains can growth in highly acidic pHs, as shown by Hong-jun et al. (2021) working with *Trichoderma guizhouense* at an optimal value of 3.0.

**C/N ratio:** C/N ratio is not frequently considered in optimisation processes, as shown in Table 1.4. Recent studies have obtained increased conidia production when fermenting high C/N ratio substrates, implying that high carbon ratios are beneficial for conidia production (Mishra et al. 2016) until a maximum C/N ratio is reached (Sharma et al. 2002). In *Trichoderma* spp., sporulation is highly influenced by the nature of carbon and nitrogen sources (Verma et al. 2007). A minimum C/N ratio value of 14 was presented by De la Cruz Quiroz et al. (2015), stating that higher values favour sporulation induction, and also presenting starchy substrates as the best for conidia production due to their adequate C/N ratio. Barrera et al. (2019) obtained an optimal C/N ratio of 18 using *Trichoderma asperellum* and polyurethane foam as support. However, Safavi et al. (2007) found that conidia produced within lower C/N ratios were generally more virulent.

It is also relevant to note that many substrates, particularly lignocellulosic wastes, can be slow or non-biodegradable carbon sources. In these cases, the use of a C/N ratio based on biodegradable organic carbon would be more adequate, as it highly differs from the ratio solely based on chemical analyses (Puyuelo et al. 2011).

**Nutrient supplementation:** it is often used to enhance conidia production, even though it is not majorly implemented as shown in Table 1.4. Recent studies have achieved higher conidia production by supplementing their substrate/media with nutrients (Mishra et al., 2016; Balakrishnan et al., 2011; Jin et al., 2016). However, nutrient supplementation is not always needed to achieve sufficient spore production yields (Cavalcante et al. 2008; Mar and Lumyong, 2012). In fact, it is one of the often-ignored parameters in SSF processes optimization, mostly due to the rise in costs that might

represent, except when nutrients are provided adding a supplementary substrate, which might ideally be an agro-industrial waste.

## 1.4. Main challenges on fungal SSF conidia production

Although fungal SSF research has not stopped growing and its tendency seems to continue rising in the next years (as shown in Figure 1.3), most of the efforts have still been done at laboratory scale. Most of them are not intended to end up as feasible solutions at larger scales, manifesting a huge gap between laboratory and industrial application. As shown in Table 1.4, most of the presented studies have been done at laboratory scale, highlighting the need of more research studies to develop fungal SSF industrial processes. To follow this direction, several challenges must be addressed.

In the frame of a circular economy and in a world where food waste has arisen as an enormous problem (it is estimated that around 1/3-1/2 of the world food production is not consumed) (Russ and Schnappinger, 2007; Stenmark et al., 2016), SSF fungal fermentation must be directed to the priority use of agro-industrial wastes (or food waste) as substrates. In some countries, a tendency shift has become urgent, as nowadays most fungal conidia production is still performed using cereal grains as major substrate (Mascarin et al., 2019). Companies should focus on using waste as substrates for fungal SSF. However, more research in SSF conidia production must be conducted to reach this point. This research must be focused on three key points, which are substrate selection, process optimization and process scale-up.

As it has been presented in Table 1.3, a vast majority of agro-industrial wastes have been tested for fungal SSF conidia production with various strains. However, not all these SSF processes have been optimized, even at laboratory scale, hampering their potential to be scaled-up to bench or industrial scale. This optimization must be carried on from the moment of the substrate selection, aiming to obtain high productivities from the very initial tests. As presented in section 1.3, sufficient knowledge on the effect of some relevant parameters in SSF processes is still lacking. Although some of the parameters mentioned in section 1.3.4 are known to have superior impact/relevance on fungal SSF conidia production in comparison to others, none of them should be optimized without taking into consideration its effect on the others, adding difficulties on which parameters should be optimized in each SSF process. A careful selection must be done, mostly depending on the nature of each process and its final requirements.

The other inherent problem within all SSF processes lays on their scale-up. As previously stated, numerous problems must be addressed when scaling-up fungal SSF processes. Several constraints regarding the scale-up effect are present, mainly related to heat and mass transfer phenomena presented in the solid-liquid interphase (Cerda et al., 2017; Soccol et al., 2017; Krishania et al., 2018), thus difficulting the application of the technology at industrial scale. In addition, when addressing fungal SSF scale-up, a shift concerning used reactor configurations is needed, with the aim to achieve higher productivities when comparing with the conventional ones. As stated in section 1.3.3, many industrial SSF fungal production processes are still based on bags or trays. In the last years, more emphasis has been given to column configurations and packed bed reactors, mostly due to their superior achievable productivity, and as shown in table 1.4, new reactor configurations, mostly based on columns or trays, are arising at laboratory scale. However, research on them is still scarce. Among them and with the aim of future industrial implementation, packed bed bioreactors should be further investigated due to their potential application beyond laboratory scale (Méndez-González et al., 2020; da Cunha et al., 2020).

To sum up, the need to further develop both sustainable and scalable fungal SSF processes is currently ongoing, yet still at earlier development phases. Combined potential of suitable substrates and reactor configurations with optimized operational strategies must be studied to achieve an optimized process which maximizes productivity while maintaining a sustainable approach.

# Chapter 2

Research objectives

This research has been developed within the MINECO/2016/BIOPRO project (Optimization strategies for the production of bioproducts using organic wastes by solid-state fermentation)" of the composting research group (GICOM) at UAB. The consolidated background of GICOM on the valorization of organic wastes was used as the starting point of this study. Although the group had previously worked on the production of biopesticides through SSF processes, works were focused on the development and scale-up of *Bacillus thuringiensis*-based fermentations. This is the first work on the research group based on the production of fungal biopesticides by SSF, both for BB and TH.

The main objective of this research was to study the production of a biopesticide based on conidia obtained by solid-state fermentation of agro-industrial wastes produced using either BB or TH, determining the possibilities of process scale-up.

To achieve this main goal, the following specific objectives have been developed:

- To validate and optimize SSF parameters at lab-scale in packed bed batch systems for both BB and TH.
- To identify the adequate substrates out of several agro-industrial residues at lab-scale
- To understand the role and relevance that several parameters play on BB and TH conidia production by SSF.
- To test novel SSF conidia production strategies based on packed bed reactors at laboratory scale, with the aim of maximizing conidia productivity.
- To scale up the most feasible strategy(s) up to 22 L, with the aim of maintaining conidia productivity while overcoming major SSF scale-up drawbacks.
- To determine the feasibility of performing the SSF process in tray bioreactor in a preliminary attempt using same residues and fungi than in packed bed reactors.
- To test the virulence of the BB fermentation product(s) against insect pest(s) at laboratory scale.

# Chapter 3

Materials and methods

## 3.1. Materials

### 3.1.1. Fungal strains

Two strains were obtained from Colección Española de Cultivos Tipo (CECT): *Beauveria bassiana* (BB) (CECT 20374) and *Trichoderma harzianum* (TH) (CECT 2929). As established by the strains' provider and as shown in Figure 3.1, the original lyophilized strains were recovered using liquid medium (potato dextrose for BB, malt extract for TH) and cultured in potato dextrose agar (PDA) (BB) or in malt extract agar (MEA) (TH) at 30°C for 6–8 days. After fungal growth and conidiation, conidia were extracted using 10 mL of liquid medium and preserved at −80°C in sterile cryovials containing 10% (v/v) glycerol. More information on the cryopreservation and fungal culture preservation can be found elsewhere (Simione, 2009; Humber, 2012).

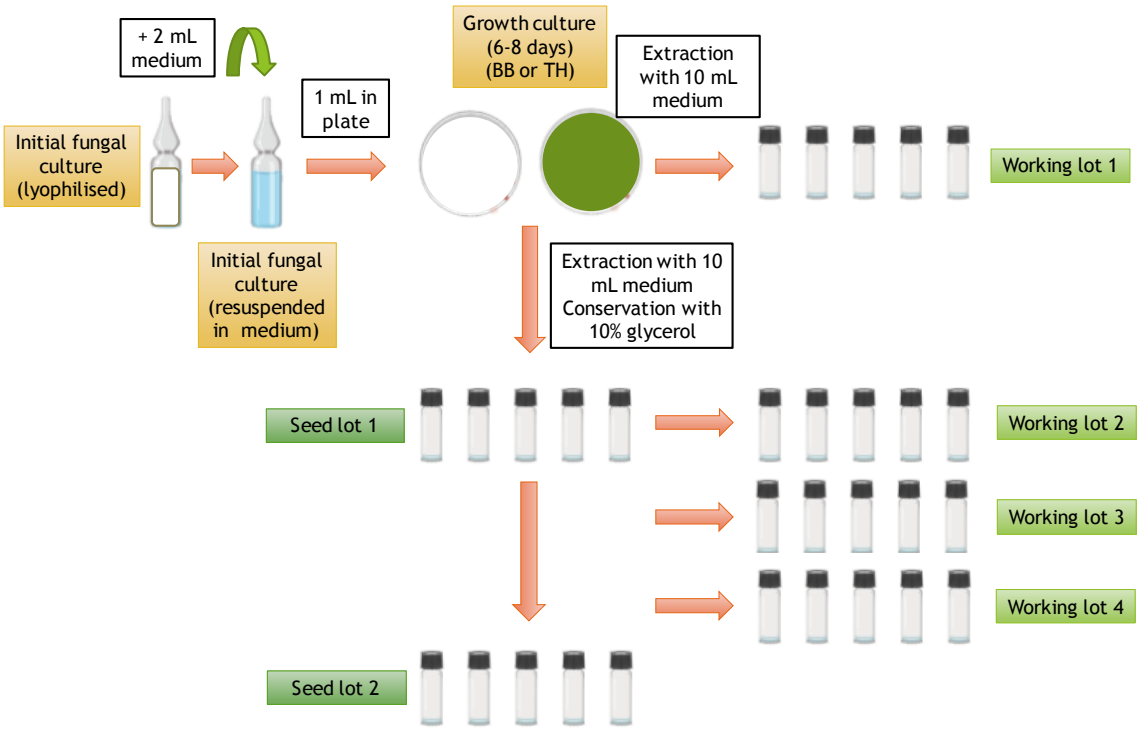



**Figure 3.1.** Lyophilized strains recovery and conservation. Adapted from Simione (2009). Some icons were provided by BioRender (https://biorender.com/).

### 3.1.2. Inoculum preparation

In each fermentation test, 1 mL of working lot was cultured using PDA or MEA at 25-30ºC. After fungal growth and conidiation occurred (6-8 days for both strains), aerial conidia were harvested using 10 mL Tween 80 dilution (0.1% for BB or 0.01% for

TH). Conidia in the suspension were counted using Neubauer chamber (Brand™ 717805) (see 3.3.1) and diluted to the appropriate concentration for each test using Tween 80 dilution at the same concentration used in conidia harvesting. Inoculum quality was visually controlled by means of optical microscope at 100x augments (Zeiss Axioskop). Typical culture growth in plates for BB in PDA and TH in MEA are shown in Figure 3.2.

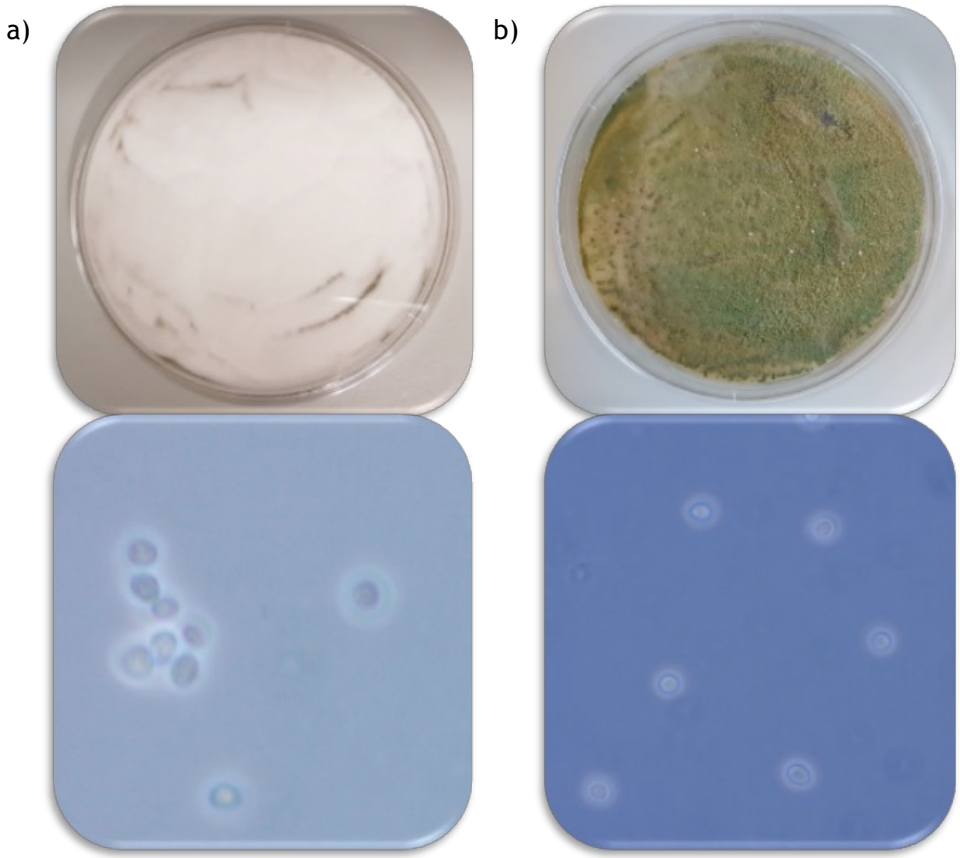



**Figure 3.2.** Plate growth and conidia visualization at 100x augments (diluted to $1x10^7$ conidia $mL^{-1}$) of BB (a) and TH (b).

### 3.1.3. Substrates

A total of nine agro-industrial wastes were used as substrates for fungal growth and conidiation. All substrates were autoclaved using disposable autoclaving bags (Dominique Dutscher, France). All substrates went through three autoclaving cycles (121ºC for 30 min) before fermentation except on Chapter 4 tests, where only one autoclaving cycle was provided. Characterization of each substrate is presented in the correspondent results Chapter.

Rice husk was used as substrate for fungal growth and conidiation in optimization and screening tests (results Block 1) and in process scale-up (results Block 2). It was

supplied by Husk Ventures S.L. (Barcelona, Spain) and stored at room temperature (20-25ºC) before use. A total of 6 different rice husk supplies were used throughout the tests. Rice husk moisture was adjusted to appropriate values in all tests before inoculation and autoclaving, considering the 10% inoculation volume in the initial moisture calculations. Volume of added water was calculated following Equation 3.1.

$$Hf = \frac{X + M \cdot Hi}{M + X} \quad (3.1)$$

Where:

Hf: final substrate moisture after water addition (%)

X: water volume to add to obtain the desired Hf value (mL)

M: initial substrate mass (g)

Hi: initial substrate moisture (%)

Beer draff was used as substrate in substrate screening (Results block 1, Chapter 5) and in process scale-up (Results block 2). It was supplied by Cervesa del Montseny S.L. (Sant Miquel de Balenyà) and stored frozen before use. A total of 3 different beer draff supplies were used throughout the tests. Beer draff' moisture and $AFP_R$ were adjusted to appropriate values in all tests by mixing the substrate with the necessary quantity of wood chips (Acalora, Ivars d'Urgell) before inoculation and autoclaving.

In the substrates screening tests (Results Block 1, Chapter 5), apart from rice husk and beer draff, six different agro-industrial wastes were also tested as substrates: apple pomace from juice production (Mooma, Fontanilles), whisky draff (local Scottish distillery), soy fiber and rice fiber from vegetable beverages industry (Liquats Vegetals S.L., Viladrau), wheat straw (UAB experimental farms), orange peel (from orange juice machine at the bar of Escola d'Enginyeria at UAB) and potato peel (Patatas Torres, Montmeló). All of them were stored frozen before use except for wheat straw, which was stored at room temperature (20-25ºC) due to having nearly no water content as rice husk. Some substrates were mixed with rice husk to enhance their $AFP_R$ or to modify other initial parameters such as moisture, the proportions are shown within their characterization in the correspondent results chapter. When working with wheat straw, initial moisture was adjusted in the same way as when working with rice husk. When working with apple pomace and orange peel, substrates were grounded before use. Initial values for all tests are given in each correspondent results chapter. If not stated otherwise,

differences in initial values between BB and TH batches in each test have not been significant.

Pre-fermentation appearances of all substrates are shown in Figure 3.3.

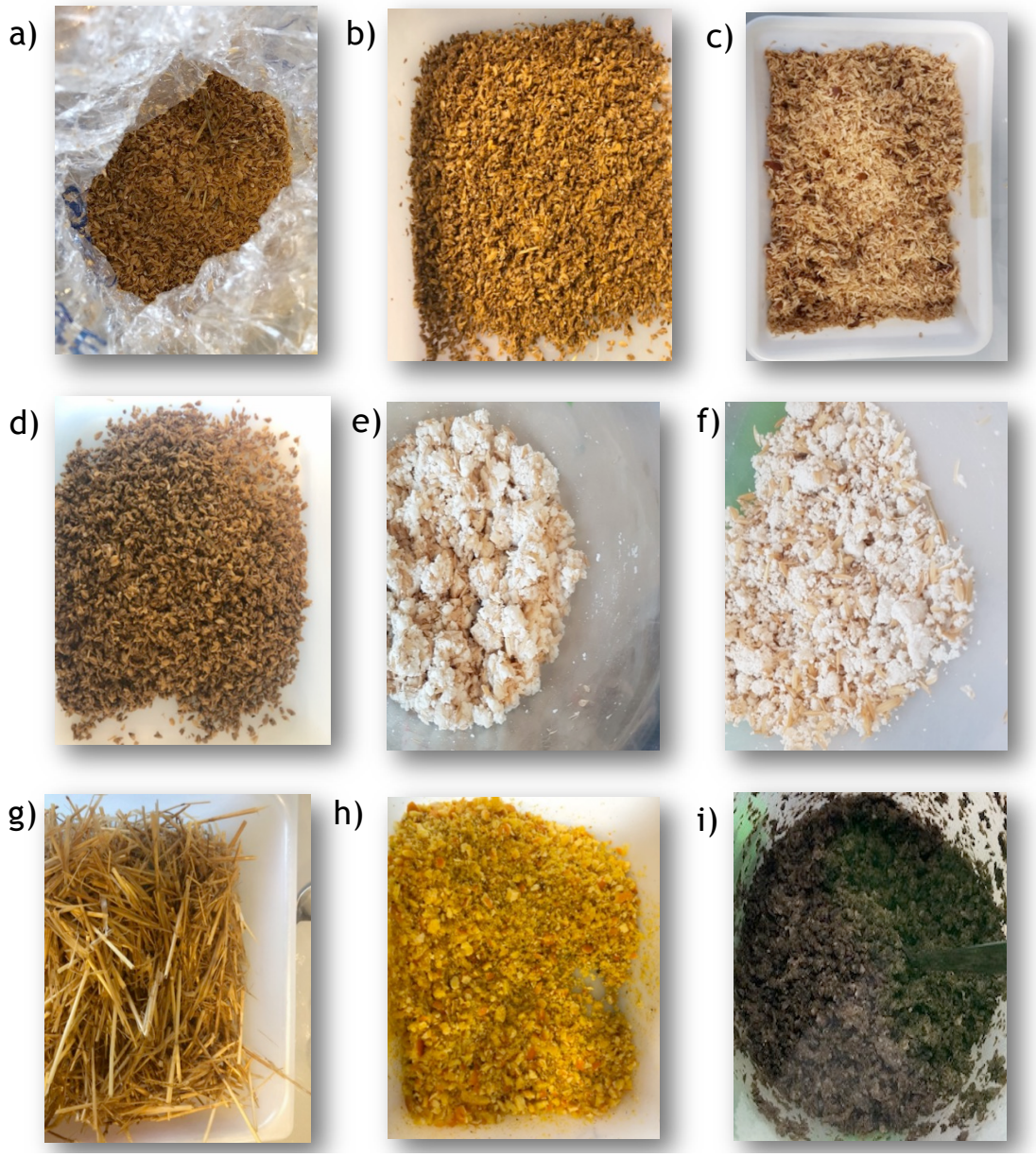



**Figure 3.3.** Pre-fermententation appearance of all used agro-industrial wastes: rice husk (a), beer draff (b), apple pomace (c), whisky draff (d), soy fiber (e) and rice fiber (f). Raw material (left) and after autoclaving (right) appearance of all used wastes: wheat straw (g), orange pomace (h) and potato peel (i).

## 3.2. Solid-state fermentation systems

Inoculum volume in each reactor in all SSF tests was 10% of its total volume, (Xie et al., 2016; Pham et al., 2010; Yang et al., 2010). When scaling, the followed criterion was superficial velocity per gram of dry matter ($m^3\ s^{-1}\ g^{-1}dm$), following Equation 3.2:

$$V_{phase} = \frac{Q}{A} \tag{3.2}$$

Where:

$V_{phase}$: superficial velocity ($m^3\ s^{-1}\ g^{-1}dm$)

Q: airflow per gram of dry matter ($m^3\ g^{-1}dm\ s^{-1}$)

A: reactor area ($m^2$)

### 3.2.1. SSF system 1: 0.5 L scale

SSF system 1 was designed, built and well described in numerous previous works of the GICOM group, the first ones being Ballardo (2016) and Cerda (2017). Briefly, this system allows to perform SSF tests at 0.5L scale in a controlled environment in terms of both $O_2$ supply (on-line monitoring of the process through respirometry) and temperature (controlled by water baths). Constant airflow was provided by means of a mass flowmeter (Mass-Stream D-6311, Bronkhorst, Netherlands) and humidified before entering the reactors from the bottom, forcing it to flow through the substrate across all the reactor's height until reaching the top of the reactor. Exhaust gases flow through a vapor trap to prevent excess water from reaching the $O_2$ sensor. The oxygen percentage in the output gases was measured by an electrochemical O2-A2 oxygen sensor (Alphasense, UK) connected to an on-line self-made data acquisition system (Arduino®-based), that recorded $O_2$ concentration and calculated the respiration rates as shown in section 3.3.2. Air supply and data acquisition system were the same in all SSF systems.

The reactors consisted of a polyvinyl chloride (PVC) cylinder of 13 cm height and 7 cm diameter, corresponding to a working volume of 0.45 L and a total volume of 0.5 L. All tests were performed using approximately 100 g of substrate. To prevent possible contamination of the material after autoclaving, all reactors were always loaded inside a laminar flow chamber. Previously they were cleaned with water and bleach, as they are PVC-made out and they could not be autoclaved.

The experimental setup of SSF system 1 for one reactor is shown in Figure 3.4.

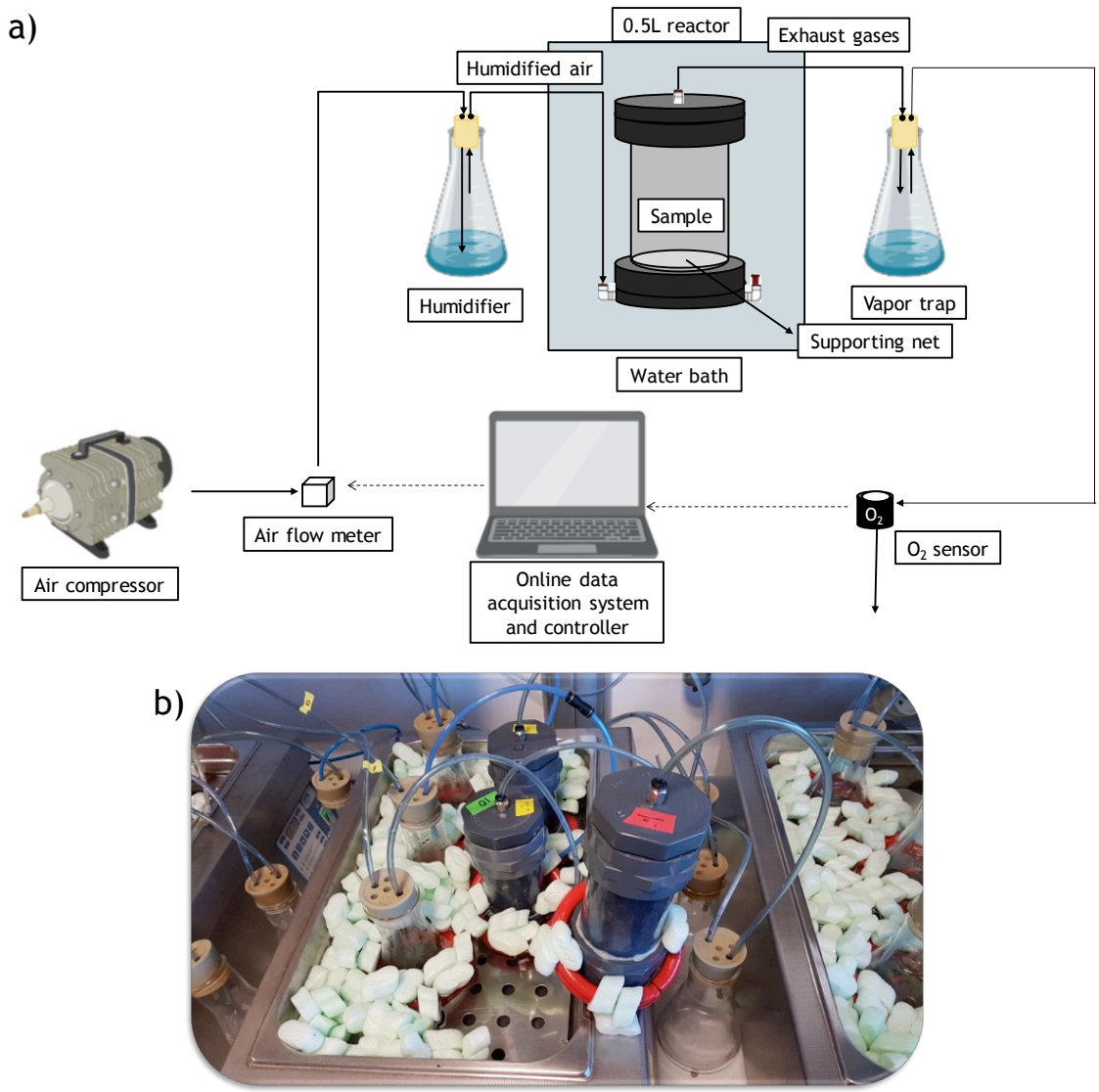



**Figure 3.4.** Experimental set-up of SSF system 1 (a) and bioreactor appearance (b).

### 3.2.2. SSF system 2: 1.5 L scale

SSF system 2 consisted of polyvinyl chloride (PVC) cylindrical reactors of 21 cm height and 10.5 cm internal diameter, corresponding to a working volume of 1.35 L and a total volume of 1.5 L.

All tests run in SSF system 2 were performed with a 300 g of non-inoculated substrate load. Temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) and a temperature probe were used to obtain accurate temperature profiles at different reactor heights, its distribution is shown in section 3.3.4. Reactors were loaded and mixed with the appropriate volume of inoculum in 3 rounds of 100 g to ensure a homogeneous distribution of the inoculum while maintaining sterile conditions.

Rest of the test performance was identical to SSF system 1. Reactor set up is shown in Figure 3.5

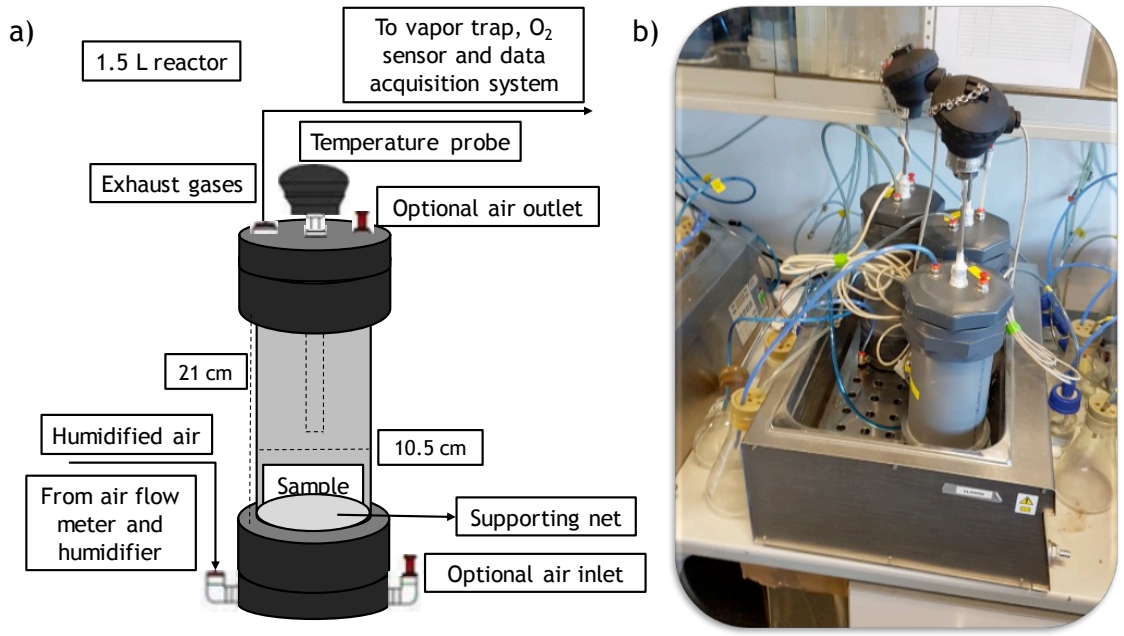



**Figure 3.5.** Reactor set-up of SSF system 2 (a) and bioreactor appearance (b).

### 3.2.3. SSF system 3: 22 L bioreactor

SSF system 3 consisted of a cylindrical stainless-steel reactor with a total volume of 22 L with a removable inner basket (dimensions were 48 cm height x 24.5 cm diameter). 3000 g of non-inoculated substrate (rice husk) or 4000 g (beer draff + wood chips) were loaded into the basket (corresponding to 90% of its working volume) in each batch. The reactor set-up is shown in Figure 3.6.

Temperature of the solid media was monitored on-line in the lower half of the bed by means of a temperature probe (Pt-100 sensors, Sensotrans). A temperature profile at different heights of the bed was obtained, complete sensor distribution is shown in section 3.3.4. To work in conditions as sterile as possible, the reactor was cleaned with water, bleach and ethanol before and after every batch. Inoculation was performed in previously cleaned vessels before loading the substrate into the basket, in a maximum of 500 g of non-inoculated material per round to ensure homogeneous distribution of the inoculum throughout the packed bed. If not stated in the correspondent results and discussion section, substrate inoculation was not performed under sterile conditions.

Final samples of 22 L reactors were divided in three areas (superior, central or lower) depending on their axial position in the reactor's bed (28–40, 12–28 and 0–12 cm

height). Analytical methods were analysed at different reactor heights, providing statistical difference between different reactor areas. Schematic representation of the sampling areas is presented in Figure 3.7. Specific analyses are detailed in Chapter 6.

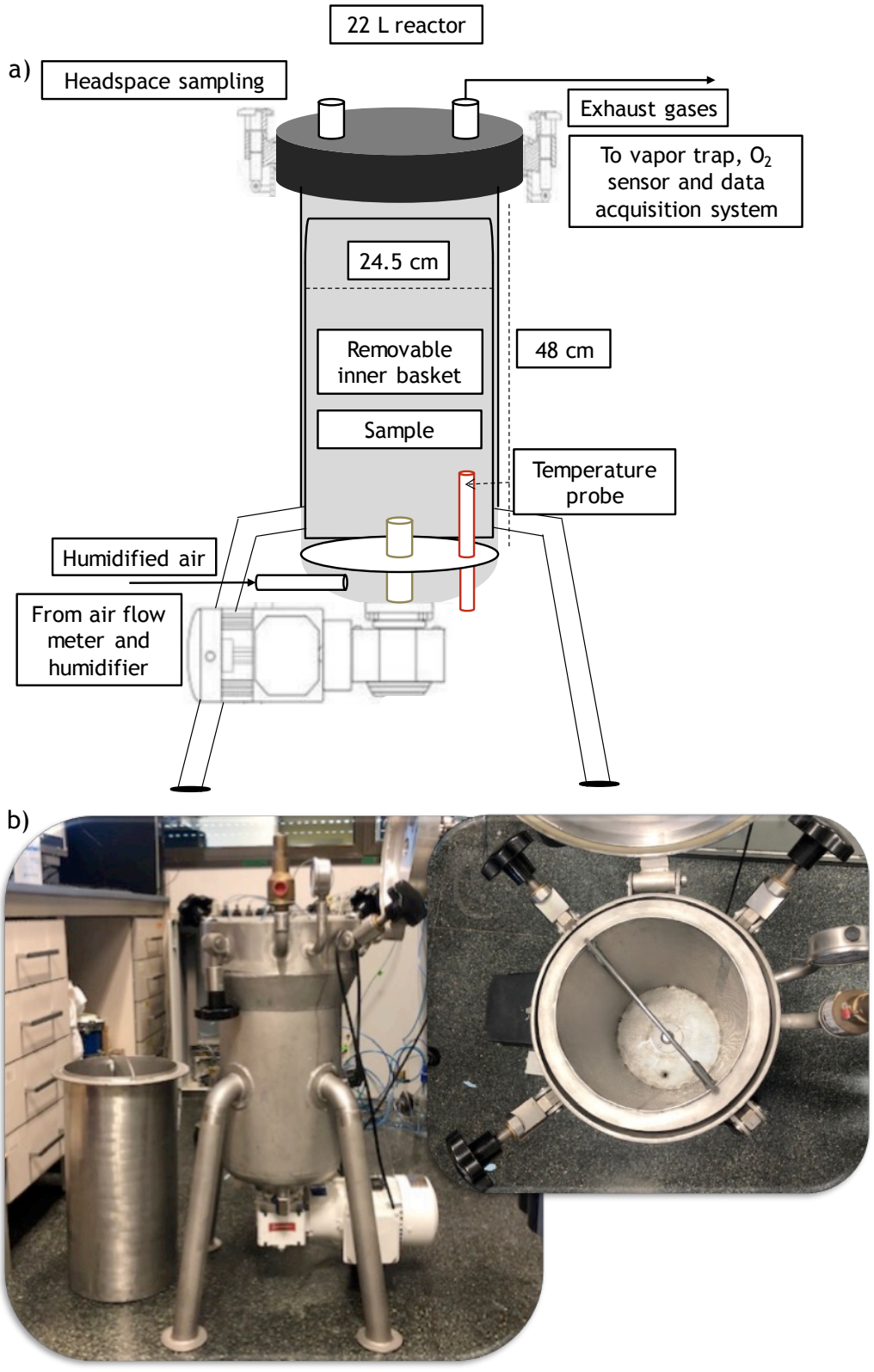



**Figure 3.6.** Reactor set-up of SSF system 3 (a) and bioreactor appearance (b).

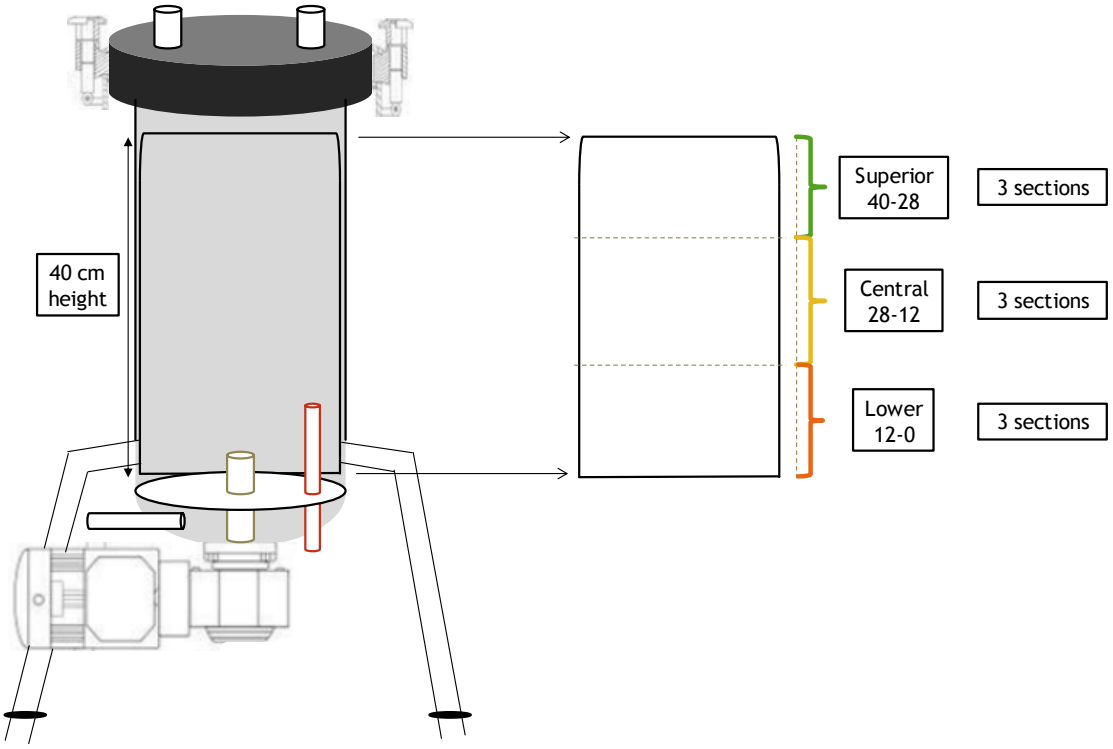



**Figure 3.7.** Schematic representation of 22 L reactor final sampling areas.

### 3.2.4. SSF system 4: tray bioreactor

SSF system 4 consisted of a tray bioreactor adapted from an incubator (Memmert ® GmbH + Co.KG P.O. Box 1720 91107 Schwabach Bundesrepublik Deutschland / Germany) with 2 or 3 trays, as presented in Figure 3.8 (a and b). Each tray dimensions were 39.5 cm length x 27.5 cm width, with 4 cm substrate bed height in all tests. Trays' bottom was perforated to ensure proper air distribution throughout the reactor. Four sprinklers were used to ensure proper air distribution. Two set-ups were used depending on the air sprinklers' disposition: while in set-up a sprinklers were facing the trays, in set-up b sprinklers were facing the bottom of the reactor to improve air circulation. Bioreactor appearance when charged with the substrate is also shown in Figure 3.9 (a and b).

When working with rice husk, 450 g of non-inoculated material were loaded per tray. When working with beer draff, 500 g of non-inoculated material were loaded per tray when inoculating BB and 750 g when inoculating TH. When obtained, respiration profiles correspond to total oxygen consumption presented by all trays in the tray bioreactor, as it was not possible to adapt the system to obtain respiration data corresponding to each individual tray. Adsorbent material (Vileda Professional, Freudenberg Home and Cleaning Solutions Ibérica, S.L.U.) was added to the top of the reactor (set-up b) in order to prevent water from exhausted air to drop onto the closest

tray. Temperature profiles were obtained in all trays for different positions, complete distribution of the temperature sensors is shown in section 3.3.4. Prior to all tests, both incubator and trays were cleaned and inoculated using the same method presented for SSF system 3.

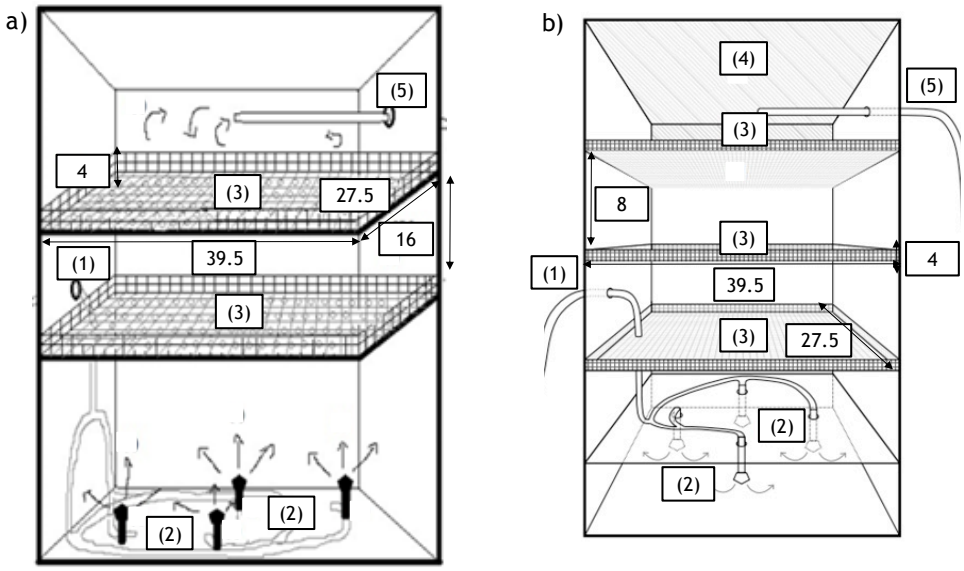



**Figure 3.8.** SSF system 4 reactor set-up of. Set-up with two trays (a). Set-up with 3 trays (b). In each set-up: air inlet (1); air sprinklers (2); trays with 1 cm diameter holes in the bottom (3); adsorbent (4) (only design b) and air outlet (5). Adapted from Echegaray (2020) and Palomas (2021).

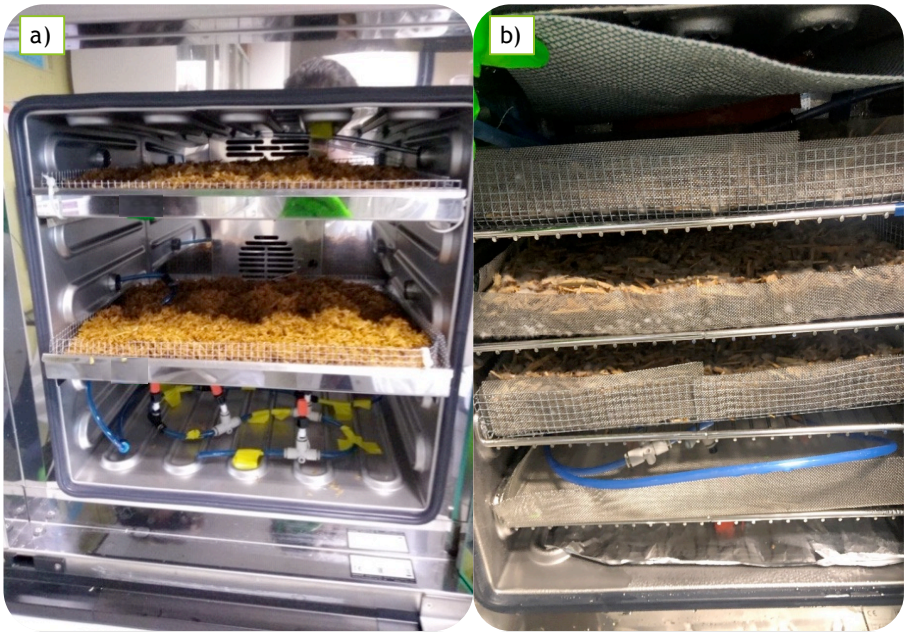



**Figure 3.9.** Reactor appearance of SSF system 4. Two tray bioreactor appearance (a) and three tray bioreactor appearance (b).

## 3.3. Analytical methods

### 3.3.1. Conidia extraction and counting

Neubauer chamber (Brand™ 717805) was used to determine fungal conidia concentrations. Conidiated substrate samples were mixed with Tween 80 solution (0.1% for BB or 0.01% for TH) in a 1:5 (v:v) proportion, shaken at room temperature in a shaker/incubator for 20 minutes (ZWYR-200D, Labwit Scientific) and diluted before counting. All conidia counts were performed per triplicate and related to the dry matter present in the reactor at counting time, following Equation 3.3:

$$\text{Conidia concentration} = \frac{\text{Nº of conidia}}{\text{CV} \cdot \text{DF}} \cdot \frac{EV}{DM} \quad (3.3)$$

Where:

Conidia concentration (conidia $g^{-1}$dm)

Nº of conidia: the counted conidia in the Neubauer chamber at a known dilution

CV: Neubauer chamber counting volume (mL)

DF: dilution factor of the counting tube

EV: dilution volume (mL)

DM: sample dry matter (g dm)

Conidia counting was performed following guidelines by Bastidas (2009). Briefly, 10 µl of the sample diluted at an appropriate conidia concentration (between 250.000 and 2.5 million conidia per mL) were loaded into the Neubauer chamber. The volume corresponding to the center square (0.0001 mL) was counted using optical microscope (Olympus BH2), and the number of conidia was then transformed following equation 3.3. Figure 3.10 shows the Neubauer grid and the counting area in detail.

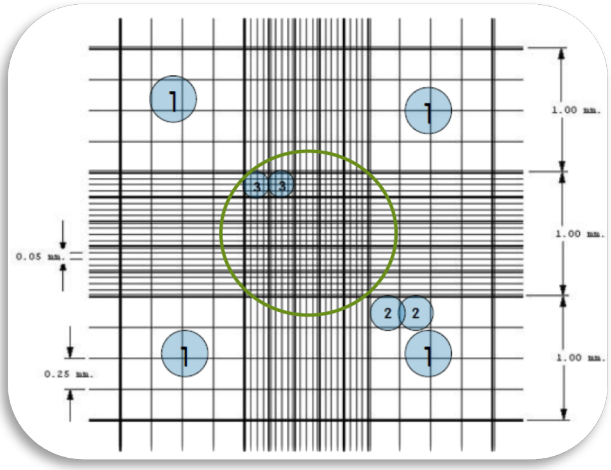



**Figure 3.10.** Neubauer chamber cell grid. Central counting area is highlighted. From Bastidas (2009).

To provide a fast comparison between conidia productions, specially between tests presenting differences in inoculum concentrations (IC), conidia quotient (CQ) was used. It was calculated as presented in Equation 3.4.

$$CQ = \frac{CC}{IC} \tag{3.4}$$

Where:

CQ: conidia quotient

CC: obtained conidia concentration in the reactor (conidia $g^{-1}$dm)

IC: inoculum concentration at the start of the fermentation (conidia $g^{-1}$dm)

### 3.3.2. Respiration indexes

Specific oxygen uptake rate (sOUR) and cumulative oxygen consumption (COC) have been used as an indirect measure of the biological activity. OUR was calculated according to Puyuelo et al. (2010), expressed as 1h average value (sOUR) (Equation 3.5) and recorded on-line:

$$sOUR = F \cdot (0.209 - yO_2) \cdot \frac{P \cdot 32 \cdot 60 \cdot 10^3}{R \cdot T \cdot DW \cdot 10^3} \tag{3.5}$$

Where:

sOUR: specific Oxygen Uptake Rate (g $O_2$ $kg^{-1}$ dm $h^{-1}$)

F: airflow (mL $min^{-1}$)

$yO_2$: oxygen molar fraction in the exhaust gases (mol $O_2$ $mol^{-1}$)

P: pressure of the system assumed constant at 101325 Pa

32: oxygen molecular weight (g $O_2$ $mol^{-1}$ $O_2$)

60: conversion factor from minute to hour

$10^3$: conversion factor mL to L.

R: ideal gas constant (8310 Pa L $K^{-1}$ $mol^{-1}$)

T: temperature at which F is measured (K)

DW: initial dry weight of solids in the reactor (g)

$10^3$: conversion factor g to mg

The area below the $O_2$ consumption curve (computed through the numerical integration in time) was used to determine the COC.

### 3.3.3. Total sugar content analysis

Total sugar content (TSC) was estimated using the anthrone method (Scott and Melvin, 1953). Sugars were extracted from dry solid samples by mixing a known amount of sample with distilled water in a 1:10 (w/v) ratio. The mixture was incubated (15 min, 50ºC) in a shaker/incubator (ZWYR-200D, Labwit Scientific) and the supernatant was recovered and centrifuged (10 min, 4000 rpm). Supernatant after centrifugation was separated and whole extraction process was repeated two more times. The total volume of the recovered supernatant was filtered with a 0.45 μm membrane filter. Anthrone reagent was prepared fresh before use by dissolving 200 mg anthrone in 100 mL ice cold 95% sulphuric acid. After that, 4 mL of anthrone agent were added to 1 mL sample supernatant in 25 mL glass tubes and the mixture was heated for 8 minutes in a boiling water bath and cooled rapidly. Absorbance of the green coloured solution was measured at 630 nm using a spectrophotometer (Varian Cary50 Bio, Agilent Technologies). Distilled water was used as blank instead of 1 mL sample supernatant. Calibration curve was prepared using glucose at 6 different concentrations ranging from 0 to 0.1 mg/mL (Figure 3.11).

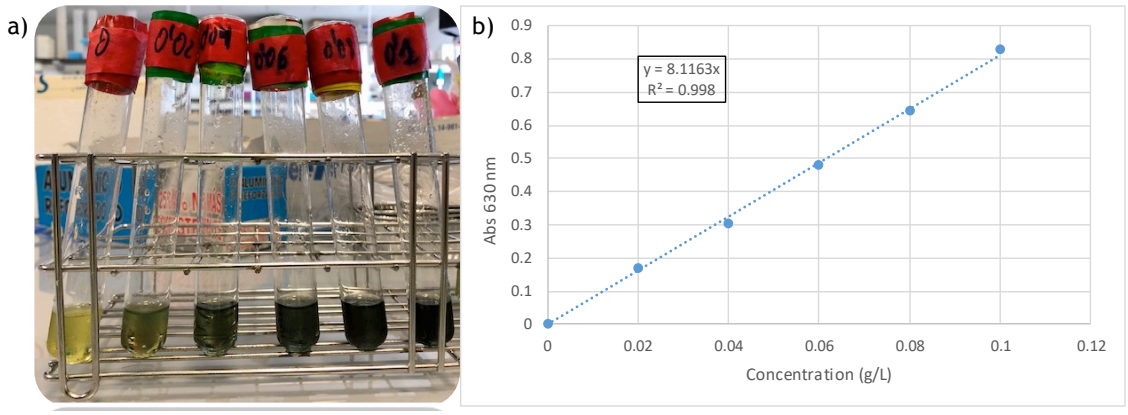



**Figure 3.11.** Visual calibration curve for total sugar content analysis (a). Example of calibration curve (b).

Total sugar content was expressed as gram of glucose equivalent per gram of dry matter according to Equation 3.6:

$$TSC = \frac{C}{P} \cdot V \tag{3.6}$$

Where:

TSC: total sugar content (g $g^{-1}$dm)

C: concentration of glucose equivalents (g $L^{-1}$)

P: weight of the dry sample (g)

V: total volume of the supernatant (L)

### 3.3.4. Temperature profiles

Temperature profiles were obtained using temperature sensors (standard Thermochron iButton device, Maxim Integrated, U.S.) distributed throughout the packed bed or in the trays as shown in figure 3.12. When working with PBBs, external sensor was used to compare with ambient temperature profile.

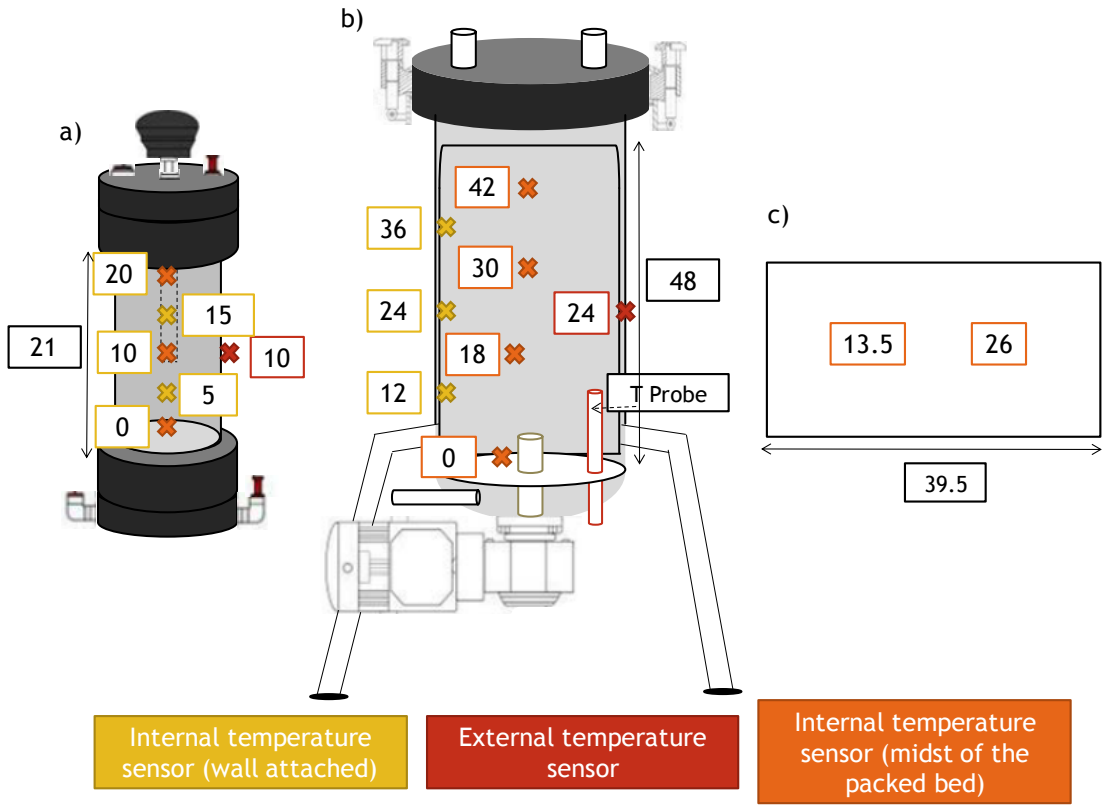



**Figure 3.12.** Temperature sensors distribution in SSF systems: system 2 (a), system 3 (b), system 4 (c). Numbers indicate height (in cm) where each sensor was located respect to reactor bottom, except for figure c) where sensor distances correspond to tray width.

### 3.3.5. Cellulase activity assay

Cellulase activity was determined in some tests performed in SSF system 1. Briefly, cellulases were extracted using 150 mL citrate buffer (0.05 M, pH 4.8) and 10 g of fermented solids in a 500 mL Erlenmeyer flask mixed through a magnetic stirrer during 30 min at room temperature. The mixture was separated by centrifugation at 10000 rpm for 20 min and by filtration with a 0.45 µm filter. The supernatant was used for cellulase activity determination (Dhillon et al., 2012). IUPAC filter paper assay was used to measure cellulase activity as described by Ghose (1987). One filter paper unit (FPU) is defined as the amount of enzyme that releases 1 µmol of glucose under assay conditions. Cellulase production has been expressed in relationship to the dry matter content of the sample (FPU $g^{-1}$dm).

### 3.3.6. Chitinase assay

#### 3.3.6.1. Reagent preparation

Colloidal chitin was used as substrate for the reaction. For its preparation, 100 mg of colloidal chitin were weighted, mixed with 1.2 mL concentrated HCl and left overnight in the fridge with magnetic stirring. The following day, 40 mL of cold EtOH were added and the obtained solution was left overnight at room temperature with magnetic stirring. The following day, the solution was centrifuged for 25 min at 6000 g. and 4ºC. Next, the supernatant was discarded. Last step was repeated by adding 40 mL of distilled water, until achieving a pH 6.0 in the obtained solution (Berna, 2012).

DNS was used as reagent to determine the absorbance activity of its reduction to 3-amino-5-nitrosalicilic acid at 540-570 nm. In a covered beaker to prevent light exposure, 60 mL of distilled water were magnetically stirred while adding 1.0 g of DNS. When dissolved, 1.6 g NaOH were gradually added. In the following 20-30 min. 30 g of Rochelle salts were slowly added. The solution obtained was then diluted to a final volume of 100 mL by adding distilled water (Miller, 1959).

Phosphate buffer was prepared by adding 3.0 g $NaPO_2$ to 400 mL distilled water in agitation. When dissolved, pH was measured and adjusted to 6.0 by adding NaOH.

Anthrone reagent was prepared by dissolving 200 mg of anthrone in 100 mL sulphuric acid 95% at close to 0ºC temperature.

#### 3.3.6.2. Activity determination

To perform chitinase activity assay, 10 g of sample were incubated in 30mL phosphate buffer pH 6.0 at ambient temperature for 2.5 h without agitation. 50 mL of the liquid extracted were mixed with 450 ml phosphate buffer 50 mM and 500 mg colloidal chitin 1% w/v. Sample was incubated for 30 min at 37ºC, 750 mL DNS were added, incubated for 10 min at 100ºC and centrifuged.

Supernatant absorbance was measured at 540 nm. Using Equation 3.7, enzymatic concentration is estimated by calibration curve.

$$Chitinase\ activity\ \left(\frac{U}{gdm}\right) = \frac{(m\Delta ABS + h) \cdot DF \cdot B}{gdm} \tag{3.7}$$

Where:

m: calibration curve slope

Δabs: sample Abs – controls Abs

DF: extract dilution factor

B: total extract volume

gdm: grams of dry matter of the initial sample

In extract control sample, colloidal chitin is replaced with phosphate buffer. In chitin control sample, liquid extract is replaced with phosphate buffer. In the blank sample, the analysis is performed using phosphate buffer.

### 3.3.7. Virulence assays

BB virulence assays were performed as part of an research stay in the University of Copenhagen (KU), Department of Plant and Environmental Science, Section for Organismal Biology (SOBI).

#### 3.3.7.1. Conidia samples

Biopesticide capabilities of the obtained lyophilised solid material fermented using BB were analysed in the KU. Samples isolated from plates were transported into conical 50 mL centrifuge tubes, while samples obtained from different SSF batches were vacuum packed after being lyophilized. The last were stored at room temperature (maximum 20ºC) before use and plate samples were stored at 4ºC.

Sample rehydration and dilution was performed using diluent Triton X 0.05%. When using BB strain KVL 13_39 from the SOBI group, strain cultivation was performed using Saboraud dextrose agar+Yeast Extract media (SDAY) diluted 4 times (SDAY/4) instead of PDA. When using BB samples obtained in the UAB, cultivation was performed using PDA.

Sample rehydration process differed depending on the samples' nature. When using plate samples, lyophilised conidia powder was rehydrated using 100 mL of Triton X 0.05%. When using solid lyophilised samples, rehydration was performed by mixing a known amount of dried sample with 5 times the volume of Triton 0.05%. Samples were agitated at 150 rpm for 20-25 minutes. Obtained suspensions were filtrated twice: first through a conventional sieve filter (around 1 mm porous) to separate big solid particles and finally through a 100 µm laboratory sieve (Endecotts Ltd, London, England). After

filtration, samples were centrifuged at 3000 rpm for 10 min using 15 mL centrifuge tubes. Supernatant was discarded and the obtained pellet was resuspended in 10 ml Triton X 0.05%. Samples were diluted to the appropriate concentration for conidia counting before germination tests (see 3.3.1) and used a maximum of 7 days after its preparation, stored at 4ºC until its use. All this process was performed in sterile conditions. Schematic presentation of sample rehydration process and spore suspension preparation is shown in Figure 3.13. This process was adapted from Inglis et al. (2012).

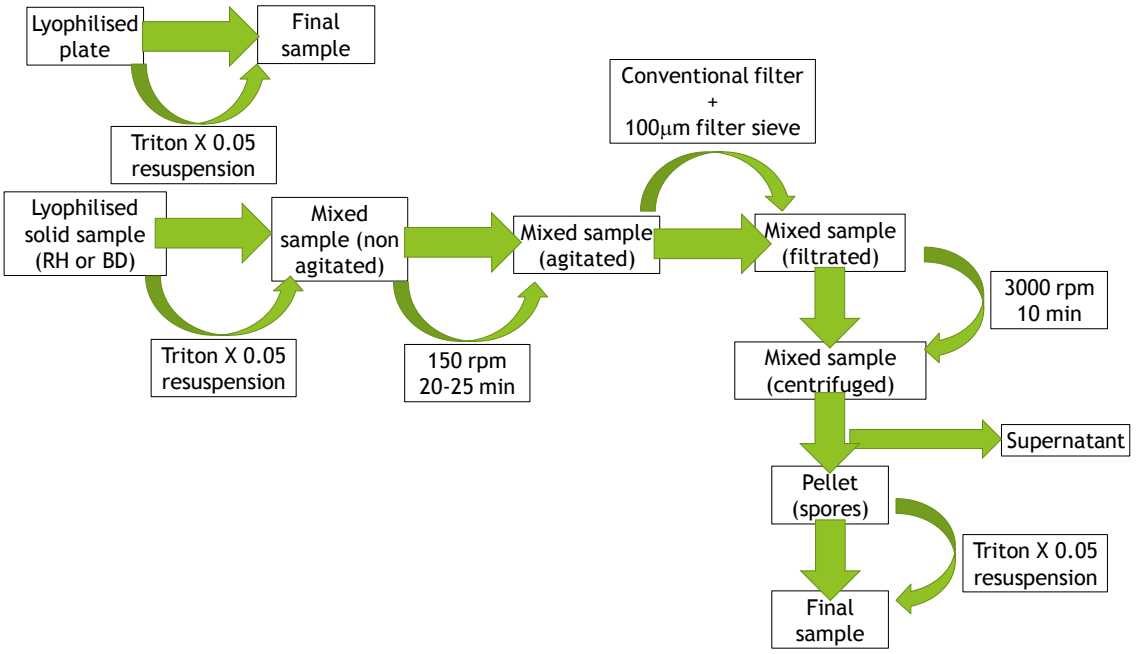



**Figure 3.13.** Sample rehydration and spore suspension preparation.

### 3.3.7.2. Conidia germination test

Conidia germination test was performed prior to the use of all suspensions in concentration and virulence assays. Briefly, after conidia counting, 100 mL of $10^6$ diluted spore suspension was transferred to a PDA (BB) plate and incubated at 25ºC for 1 day. Following, 3 cover glasses were placed in the plate and 100 conidia were counted in each cover glass, distinguishing between germinated (Gc) and non-germinated (NGc) conidia, Gc showing hyphae growth in contrast to NGc. Conidia germination (%) on each cover glass was calculated following Equation 3.8, while plate values were calculated on average.

$$CG(\%) = 100 * \frac{G_c}{(G_c + NG_c)} \quad (3.8)$$

Where:

CG: conidia germination (%)

$G_C$: germinated conidia

$NG_C$: non-germinated conidia

### 3.3.7.3. Insect culture

Insect culture consisting of larvae of the yellow mealworm Tenebrio molitor L. (Coleoptera: Tenebrionidae) was obtained from Avifauna (Denmark) and kept in darkness at constant temperature of 25ºC (Series BD Classic Line Model 400, Binder) in ventilated plastic containers (30x21x20 $cm^3$). Each container received 160-175 g of larvae, 500 g of organic oatmeal and 150-200 g slices of organic potatoes. Potato slices were replenished every 2-3 days. Pupae were collected regularly and kept separated in a medium-sized ventilated plastic box (22x17x6 $cm^3$). Upon adult emergence, beetles were separated from the pupae and maintained in a plastic container (30x20x12 $cm^3$) with oatmeal and potato slices.

For experiments using larvae, individuals were selected at 2-3 weeks after reception, measuring between 1 and 1.5 cm, colouring yellow-tan. When using adults, individuals were selected between 1-3 weeks after adult eclosion, ensuring complete black colour of cuticle.

Schematic representation of the *T. molitor* life cycle is shown in Figure 3.14.

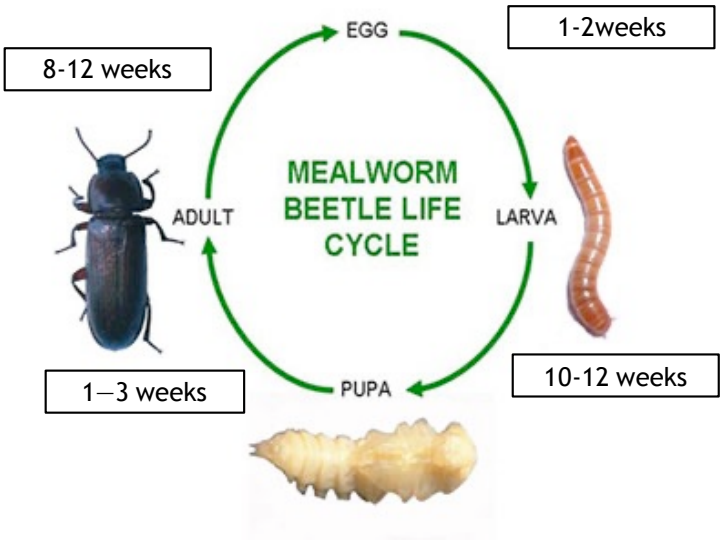



**Figure 3.14.** Schematic representation of *T. molitor* life cycle. Adapted from: blogs.edutech.nodak.edu.

#### 3.3.7.4. Assay set-up

Medicine cups of 30 mL were used as set-up for assays with individual larvae and adults.

When working with larvae, each *T. molitor* individual (previously introduced in a medicine cup) was exposed by pipetting 2 µL of the conidia suspension onto the anterior part of the body and left inside the medicine cup with a moist filter paper at 25ºC in darkness for 24 h, to maintain sufficient moisture for germination of conidia. Each cup was sealed with a lid perforated with three holes to allow sufficient airflow inside the cup. After 24 h, filter paper was removed and replaced with a 1% water agar cube (0.5-1 cm edge). Each larva was fed with 0.75-1 g wheat bran (Coop Danmark A/S, Denmark) and left in the medicine cup at 25ºC for up to 14 days. Mortality was checked daily. Dead individuals were collected and surface sterilized by immersion in 5% sodium hypochlorite for 30 s, followed by two rinses of 30 s in deionized water. The rinsed cadavers were then left individually inside parafilm-closed Petri dishes with moist filter paper inside at 25ºC for 2-7 days in order to stimulate fungal emergence from the dead insects (mycosis).

Adults of *T. moli*tor were exposed by pipetting 5 µL of the conidia suspension onto the intersegmental membrane between the head and the pronotum. Each beetle was then left at 25ºC in the medicine cup with a potato slice for 24 h in darkness, to maintain sufficient moisture for fungal germination. Potato slices were removed and replaced with moist filter paper inside the medicine cups and the beetles were fed with 1.5-2 g oatmeal and left at 25ºC for up to 14 days. Mortality was checked daily. Moist filter papers were replaced if dry. Dead individuals were collected and surface sterilized as for the larvae.

Figure 3.15 shows an example of test set-up for each *T. molitor* stage.

**Figure 3.15.** Test set-up for adult *T. molitor* stage. First column: day 0-1. Second column: day 1-14. First row: larvae stage. Second row: adult stage.

#### 3.3.7.5. Mortality correction

Abbott's formula was used to correct insect mortality in all insect assays. Control sample serves as decrease in the background population. Corrected mortality was calculated according to Equation 3.9. as presented in Capinera (2004):

$$Corrected\ mortality = \frac{\%\ test\ mortality - \%\ control\ mortality}{100 - \%\ control\ mortality} \qquad (3.9)$$

#### 3.3.7.6. Lethal time 50 ($LT_{50}$)

Time to reach 50% mortality of the total population ($LT_{50}$) was calculated by means of probit analysis (Finney, 1971).

### 3.3.8. Microbial identification

Microbial identification was performed by external analysis after isolation of obtained cultures in petri dishes. Identification was carried out in the Instrumental Techniques Laboratory (Nucleic acids analysis area) of Universidad de Leon. Briefly, qPCR (quantitative polymerase chain reaction) was performed using a thermal cycler

(GeneAmp PCR 2700 (Applied Biosystems)). PCR products were purified using a NucleoSpin Gel and PCR Clean-up (Macherey-Nagel) kits, and they were sequenced using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Microbial identification was performed by comparison of the sequences with the data bank GenBank NCBI (National Center for Biotechnology Information, using the software BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

### 3.3.9. Standard analytical methods

Regular parameters were determined according to the standard procedures included in "Test Methods for the Examination of Composting and Compost" (US Department of Agriculture and The US Composting Council, 2001). All the results were calculated as a mean of three replicates and presented as mean ± standard deviation.

#### 3.3.9.1. Bulk density and air-filled porosity

Bulk density (BD) is defined as the weight per volume unity of a sample. BD was calculated in a wet basis and used for porosity calculations, dividing the sample weight by the sample volume according to Equation 3.10.

$$BD = \frac{P}{V} \tag{3.10}$$

Where:

BD: bulk density (g $L^{-1}$)

P: sample weight (g)

V: sample volume (L)

Air-filled porosity ($AFP_R$) is defined as the volume fraction of air (usually reported in a percentage basis) in a porous matrix. It was calculated according to Equation 3.11 as presented by Richard et al. (2004):

$$AFP_R = 1 - BD_t \left( \left( \frac{1 - DM}{D_W} \right) + \frac{DM * OM}{PD_{OM}} + \left( \frac{DM(1 - OM)}{PD_{ash}} \right) \right) \tag{3.11}$$

Where:

$AFP_R$: air-filled porosity (%)

$BD_t$: total bulk density on a wet basis (kg $m^{-3}$)

DM: dry matter on a wet basis (%)

OM: organic matter on a dry basis (%)

$D_w$: water density (1000 kg $m^{-3}$)

$PD_{OM}$: organic fraction particle density (1600 kg $m^{-3}$)

$PD_{ash}$: ash particle density (2500 kg $m^{-3}$).

Both BD and $AFP_R$ were presented as a mean of three replicates and presented as mean ± standard deviation.

#### 3.3.9.2. Moisture and dry matter

Moisture content (MC) and dry matter (DM) determination were performed by gravimetric analysis. Samples (5 to 10 g) were placed in a previously weighted crucible and left in an air oven at 105ºC for at least 24 h to ensure the drought of the sample. The crucible with the dried sample was weighted after cooling, and MC and DM were calculated following equations 3.12 and 3.13:

$$MC = \frac{Pi - Pf}{Pi - Po} \, x \, 100 \tag{3.12}$$

$$DM = 100 - MC \tag{3.13}$$

Where:

MC: moisture content (%)

Pi: initial wet weight of the sample (g)

Pf: final dry weight of the sample (g)

Po: crucible weight (g)

DM: dry matter content (%)

#### 3.3.9.3. Organic matter

Organic matter (OM) determination was performed by gravimetric analysis. 1-2 g of the obtained samples in section 3.3.2.1 were ignited in a muffle at 550ºC in the presence of excess air for at least 2 h. The remaining ashes present in the crucible were weighted after cooling and OM was calculated following equation 3.14:

$$OM = \frac{Pi - Pa}{Pi - Po}\ x\ 100 \qquad (3.14)$$

Where:

OM: organic matter content (%)

Pi: initial weight of the dry sample (g)

Pa: final weight of the ashes (g)

Po: crucible weight (g)

#### 3.3.9.4. C/N ratio

C/N analysis was performed by means of chemical elemental (C, H, N and S) analysis by Servei d'Anàlisi Química (SAQ) in UAB. The analysis was carried out using a CHNS elemental analyzer Flash 2000 (Thermo Scientific). Samples were combusted at 1200°C with air excess and quantification was performed by means of gas chromatography.

#### 3.3.9.5. pH and conductivity

pH and conductivity were determined by means of the aqueous extract obtained after mixing the sample with distilled water in a 1:5 w/v ratio. The sample was shaken at room temperature for 30 min to solubilize the salts into the supernatant. pH was measured with an electrometric pH meter (Crison®, micropH2001) and conductivity was measured using an electrical conductivity meter (XS Cond 8).

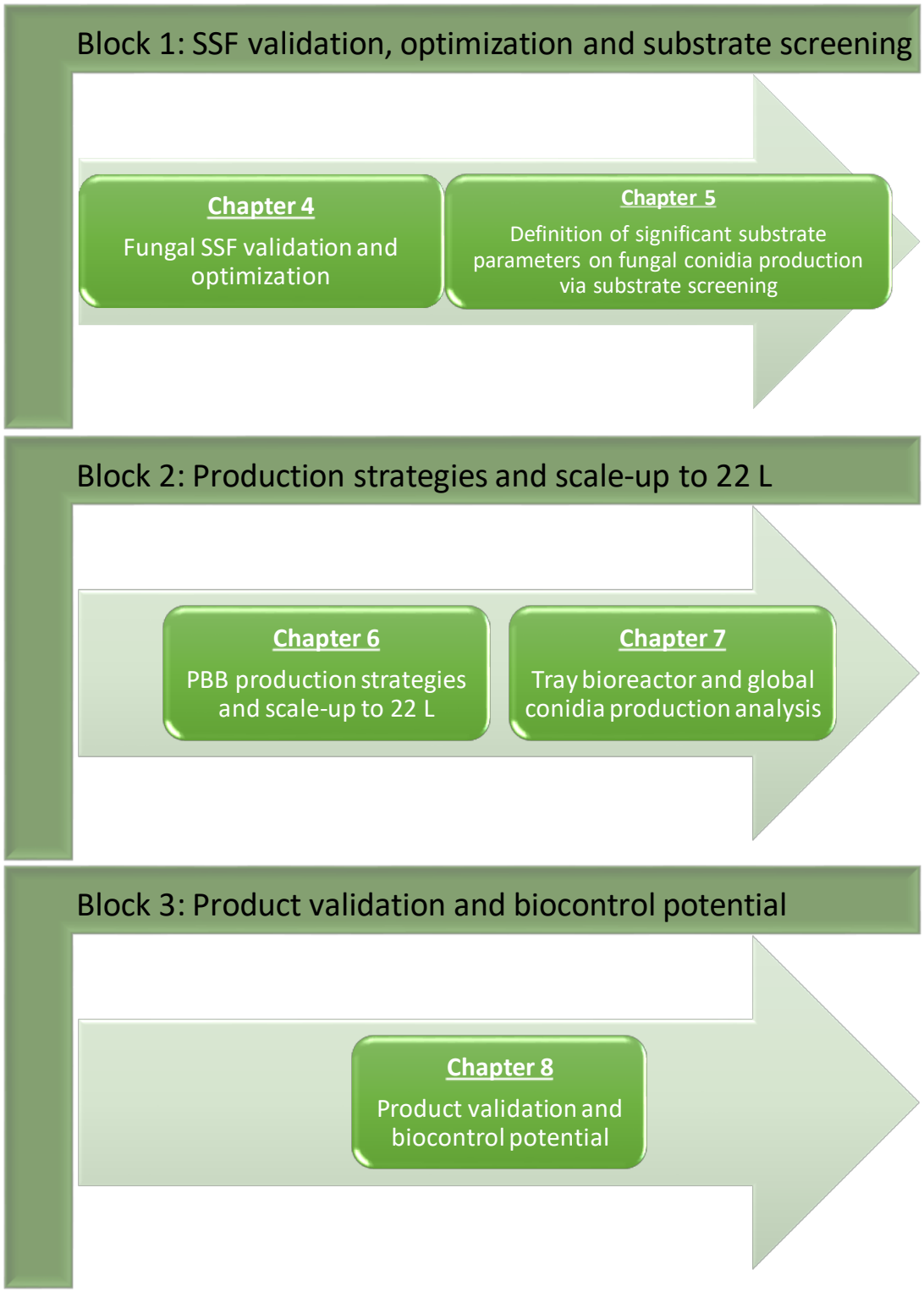
#### 3.3.9.6. Statistical analysis

Statistical difference between samples was analysed by means of a one-way ANOVA ($p < 0.05$ confidence) with the Tukey test using Minitab 17 (Minitab Ltd) software. Results were classified in letter groups. Those with different letter groups were significantly different.

# Chapters 4-8

Results and discussion

# RESULTS BLOCK ORGANIZATION



# Chapter 4

## Fungal SSF validation and optimization

Part of this chapter has been published in: Sala, A., Artola, A., Sánchez, A., Barrena, R., 2020. Rice husk as a source for fungal biopesticide production by solid-state fermentation using *B. bassiana* and *T. harzianum*. Bioresource Technology. 296. 122322.

## 4.1. Summary/Overview

In this chapter, the optimization of *Beauveria bassiana* (BB) and *Trichoderma harzianum* (TH) conidia production has been addressed using rice husk in 0.5 L packed bed bioreactors (PBBs) via solid-state fermentation (SSF).

Rice husk was chosen as sole substrate for the optimization and scale-up of a fungal SSF produced biopesticide. Prior to the start of thesis work and to the authors knowledge, few works had previously been presented using rice husk as substrate for fungal conidia production (Pham et al., 2010; Mishra et al., 2016), both with BB. To the author's knowledge, first attempts at scaling-up a SSF process using rice husk as substrate and *Trichoderma* as inoculum were performed at the same time of this thesis (Barrera et al., 2019), although with the genera *asperellum* instead of *harzianum*. Additionally, rice husk presents several characteristics which make it an interesting substrate for SSF:

- Rice husk is a by-product of rice, the most consumed cereal for a large part of humanity and the third agricultural commodity in terms of production by 2014 (Pode, 2016). In conjunction to growing human population and as shown in Figure 4.1, rice production has not stopped increasing, meaning that rice husk production is also growing every year (Food and Agricultural Organization of the United Nations, FAO). An estimated amount of 215M tonnes of rice husk were produced all over the world on 2018 (using the correlation of 0.28kg of rice husk obtained per kg of milled rice provided by the International Rice Research Institute, IRRI), making rice husk easily accessible in all countries where rice is produced.
- It is a promising substrate to overcome SSF disadvantages. In the majority of SSF processes, heat removal via convection and conduction proves ineffective, resulting in high temperatures which negatively affect microbial growth and production while also creating temperature gradients throughout the reactor bed (Pandey, 2003). Substrates which present highly porous structure such as rice husk (Phonphuak and Chindaprasirt, 2015) should help at overcoming or at least reducing this major SSF issue.
- Thanks to its naturally high air-filled porosity ($AFP_R$), rice husk can be used without mixing with a bulking agent. Being classified as a material with low potential biodegradability according to Barrena et al. (2011), air dispersion throughout the bed should be sufficient to ensure correct $O_2$ distribution and $CO_2$

removal, a crucial factor when working with PBBs. This could also help at reducing costs related to heat dispersion, which are common when scaling SSF bioreactors (Pandey, 2003).

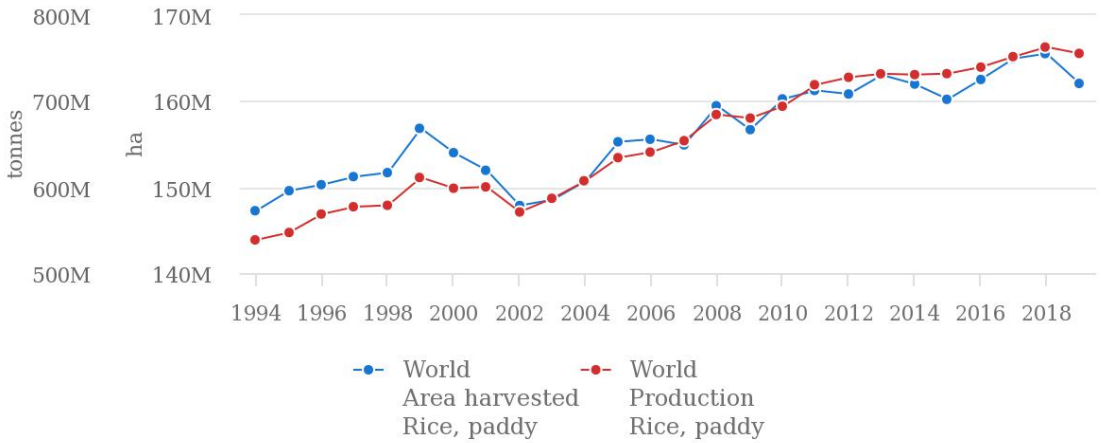



**Figure 4.1.** Production quantities and harvested area of paddy rice from 1994 to 2019. Source: FAOSTAT (consulted April 2021).

The aim of this chapter was the optimization of a SSF process for producing BB and TH conidia using rice husk as sole substrate in 0.5 L PBBs. A preliminary test on rice husk feasibility was performed with each strain prior to several optimization tests. Optimization has been focused in seven different parameters to maximize conidia production. The use of different rice husk supplies has allowed the achievement of generalized results for the substrate when operating within the tested parameter ranges. The specific objectives of the chapter are: (a) to maximize conidia production using BB and TH as inoculum by finding optimal values or suitable ranges for the main parameters affecting fungal conidia production, (b) to determine SSF robustness by determination of a reliable conidia production range by using different substrate batches and (c) to obtain crucial information for further scale-up of the process.

## 4.2. Materials

A total of 4 different rice husk supplies were used to perform all the presented tests, its characterization is presented in Table 4.1.

**Table 4.1.** Rice husk characterization for the four different supplies used in 0.5 L optimization tests.

| Parameter/RH supply | RH1 | RH2 | RH3 | RH4 | Mean values |
|---|---|---|---|---|---|
| **MC (%)** | 10 ± 0.2 | 14 ± 0.1 | 9.8 ± 0.2 | 9.9 ± 0.1 | 10.9 ± 0.2 |
| **OM (%)** | 79.6 ± 1.0 | 78.3 ± 1.2 | 83.8 ± 0.8 | 83.4 ± 1.7 | 80 ± 1.2 |
| **pH** | 6.9 ± 0.2 | 6.8 ± 0.8 | 6.8 ± 0.2 | 6.2 ± 0.2 | 6.7 ± 0.4 |
| **CD (µs·cm$^{-1}$)** | 307 ± 23 | 596 ± 43 | 847 ± 72 | 588 ± 40 | 584 ± 45 |
| **Carbon (%)** | 41 ± 0.1 | 39.1 ± 1 | 36.5 ± 0.6 | 40.4 ± 0.5 | 39.3 ± 0.6 |
| **Hydrogen (%)** | 5.2 ± 0.1 | 5 ± 0.2 | 5 ± 0.3 | 5.2 ± 0.2 | 5.1 ± 0.2 |
| **Nitrogen (%)** | 0.5 ±0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 | 0.4 ± 0.1 | 0.6 ± 0.1 |
| **Sulphur (%)** | <0.1 | <0.1 | <0.1 | <0.1 | <0.1 |
| **C/N ratio** | 78.8 ± 5.5 | 56 ± 7.4 | 53.6 ± 3.6 | 95.3 ± 13 | 70.9 ± 7.4 |
| **BD (kg m$^{-3}$)** | 165 ± 2 | 168 ± 2 | 166 ± 3 | 166 ± 1 | 166 ± 2 |
| **$AFP_R$ (%)** | 89.7 ± 1.0 | 89.3 ± 0.7. | 89.7 ± 1.1 | 89.8 ± 1.2 | 89.6 ± 1.0 |

MC: moisture content; OM: organic matter; CD: conductivity; BD: bulk density; $AFP_R$: air-filled porosity; RH: rice husk

## 4.3. Tests

### 4.3.1. Preliminary tests

A preliminary test to analyse the feasibility of rice husk as substrate for BB and TH conidia production was performed using RH1 as substrate. Triplicate fermentations were performed with both strains. Initial parameters for these fermentations are presented in Table 4.2. Except for temperature (which optimal values were provided in CECT full specification), all initial values were chosen according to data presented in bibliography (compiled in section 1.3.4 and Table 1.4).

**Table 4.2.** Initial parameter values for preliminary tests.

| Parameter | MC (%) | T (ºC) | OM (%) | IC (conidia g$^{-1}$dm) | pH | CD (µs cm$^{-1}$) | AF (mL min$^{-1}$) |
|---|---|---|---|---|---|---|---|
| **Value** | 67.5±0.9 | 25 | 80.6±1.3 | 2.1x10$^7$ | 6.8±0.3 | 332±21 | 20 |

MC: moisture content; T: temperature; OM: organic matter; IC: inoculum concentration; CD: conductivity; AF: airflow

### 4.3.2. First time course tests

Time course tests were performed with both fungal strains to establish the optimal conidia production time in terms of productivity, as well as to establish the starting point for each optimizable parameter based on bibliographical research and preliminary test. Using RH1 as substrate, six reactors with the same initial conditions were loaded. Reactor harvesting schedule is presented in Figure 4.2. A total of 20 g were extracted on the first sample of each reactor, maintaining the reactors up to a second harvest with the remaining material. On both harvests, 10 g were used for conidia count and 10 g were used to perform cellulase test. pH was also measured in all samples, whereas moisture was only measured on the initial and final samples.

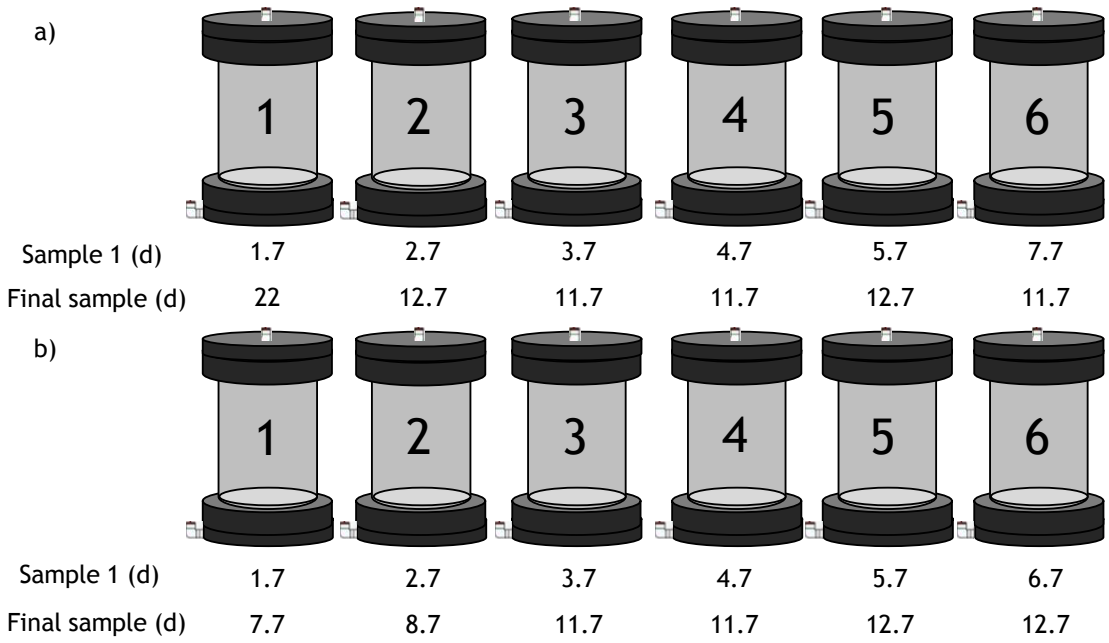
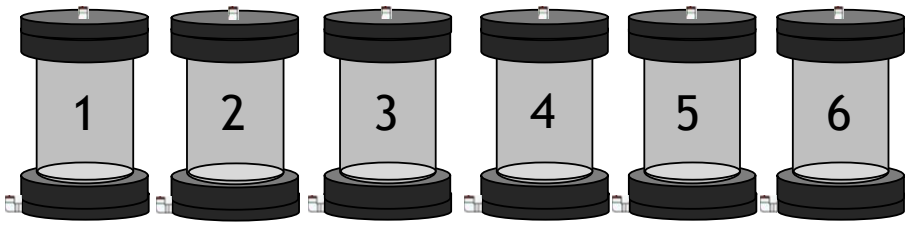



**Figure 4.2.** Reactor harvesting schedule for first time course tests. a) BB schedule. b) TH schedule.

### 4.3.3. Mixing tests

Mixing tests were performed with both strains to evaluate its effect on conidia production. Using RH2 as substrate, six reactors were harvested at the optimal conidia production time (previously found in the first time course tests). Three reactors were mixed manually inside laminar flow cabin and using sterilised tools at different times; the rest were left as control triplicates for the whole length of the test. Mixing time was established depending on optimal fungal production time found in first time course tests,

its schedule is presented in Figure 4.3. All initial parameters values were kept as presented in the first time course test.




| | | | | | | |
|---|---|---|---|---|---|---|
| BB mixing time (h) | 40 | 64 | 88 | (-) | (-) | (-) |
| TH mixing time (h) | 24 | 48 | 72 | (-) | (-) | (-) |

**Figure 4.3.** Reactor mixing schedule for mixing tests.

### 4.3.4. Designs of experiments

Two sets of experimental designs (DoEs) were performed with each strain to maximize conidia productivity at the optimal time found in the previous time course tests. Response surface analysis via two Box-Behnken designs were proposed and analyzed using the software DesignExpert 11 (Stat-Ease, Inc, United States). Each analysis was composed by 15 runs obtained after the combination of 3 numeric factors set to 3 levels. Level values (high, central and low) were chosen depending on maximums and minimums found in bibliography for each analysed parameter. A control was set in the form of a triplicate in the obtained central points. All reactors were loaded with 80 g of substrate before inoculation. Performed tests are shown in Tables 4.3 and 4.4.

#### 4.3.4.1. DoE1

DoE1 evaluated the effect of moisture, inoculum concentration and airflow on conidia production. RH2 was used as substrate in all DoE 1 tests. All performed tests in DoE1 are shown in Table 4.3.

Non analysed parameters values were initially kept as presented in the first time course, except for C/N ratio, which was the one corresponding to the raw material with no initial modifications (Table 4.1).

**Table 4.3.** DoE1 performed batches.

| Run/Parameter | MC (%) | IC (conidia $g^{-1}$dm) | AF (mL $min^{-1}$) |
|---|---|---|---|
| 1 | 45 | $1x10^6$ | 40 |
| 2 | 65 | $1x10^6$ | 40 |
| 3 | 45 | $1x10^7$ | 40 |
| 4 | 65 | $1x10^7$ | 40 |
| 5 | 45 | $5.5x10^6$ | 20 |
| 6 | 65 | $5.5x10^6$ | 20 |
| 7 | 45 | $5.5x10^6$ | 60 |
| 8 | 65 | $5.5x10^6$ | 60 |
| 9 | 55 | $1x10^6$ | 20 |
| 10 | 55 | $1x10^7$ | 20 |
| 11 | 55 | $1x10^6$ | 60 |
| 12 | 55 | $1x10^7$ | 60 |
| 13 | 55 | $5.5x10^6$ | 40 |
| 14 | 55 | $5.5x10^6$ | 40 |
| 15 | 55 | $5.5x10^6$ | 40 |

MC: moisture content; IC: inoculum concentration; AF: airflow

#### 4.3.4.2. DoE2

DoE2 evaluated the effect of temperature, C/N ratio and moisture on conidia production. Moisture was re-analyzed to observe the combined effect of it with temperature, while also trying a different range in comparison to DoE 1 tests. C/N ratio was modified by adding N supplement in the form of ammonium sulphate. RH3 was used as substrate in all DoE 2 tests. All performed tests in DoE2 are shown in Table 4.4.

Inoculum concentration and airflow were adjusted to obtained values in DoE1, the rest of the non-analysed parameters were initially kept as presented in the first time course.

**Table 4.4.** DoE2 performed batches.

| Run/Parameter | T (ºC) | C/N | MC (%) |
|---|---|---|---|
| 1 | 25 | 25 | 60 |
| 2 | 39 | 25 | 60 |
| 3 | 25 | 55 | 60 |
| 4 | 39 | 55 | 60 |
| 5 | 25 | 40 | 50 |
| 6 | 39 | 40 | 50 |
| 7 | 25 | 40 | 70 |
| 8 | 39 | 40 | 70 |
| 9 | 32 | 25 | 50 |
| 10 | 32 | 55 | 50 |
| 11 | 32 | 25 | 70 |
| 12 | 32 | 55 | 70 |
| 13 | 32 | 40 | 60 |
| 14 | 32 | 40 | 60 |
| 15 | 32 | 40 | 60 |

T: temperature; C/N: carbon/nitrogen ratio; MC: moisture content

### 4.3.5. Second time course tests

To validate the obtained conditions in the previous tests, a second time course analysis was performed with each strain applying the previous DoEs adjusted conditions except for C/N ratio, which was kept unmodified at the same value corresponding to the used raw material (Table 4.1). Using RH4, twelve reactors with the same initial conditions were analysed one per day, reactor harvesting schedule is presented in figure 4.4. Mixing was applied following results shown in mixing tests. Same analysis as in time course 1 with the addition of moisture content were conducted with each sample.

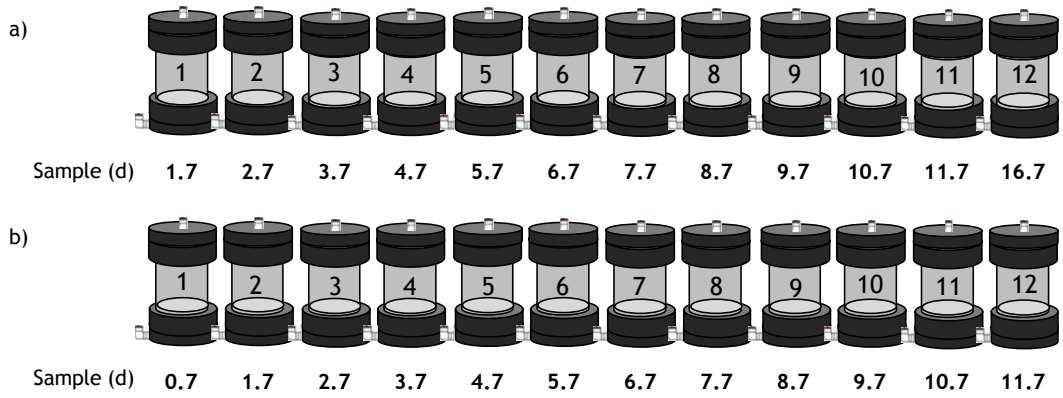



**Figure 4.4.** Reactor harvesting schedules for second time course tests. a) BB schedule. b) TH schedule.

## 4.4. Results and discussion

Results and discussion section in this Chapter is structured by presenting the results obtained on each test with both used fungal strains in the same section.

To provide a global view of the performed tests and to summarize the obtained results, all batches presented in the chapter are summarized in 3 Tables (4.7, 4.8 and 4.9) in the final section (4.5 global analysis) of this chapter.

### 4.4. 1. Preliminary tests

These tests were performed to determine the feasibility of fungal conidia production using BB and TH and rice husk as substrate.

BB conidia production was of $7.0x10^8$conidia $g^{-1}$dm, while TH conidia production was of $8.4x10^8$ conidia $g^{-1}$dm. Conidia productions were on the same order of magnitude to those found in bibliography using rice husk. With BB, Mishra et al. (2016), achieved $4.3x10^8$ conidia $g^{-1}$ using rice husk without nutrient supplementation, even though their substrate presented a lower C/N ratio (22.7 vs 78.8 in this test).

sOUR profile and conidia visualization at 100x augments for both fungi are shown in Figure 4.5. According to Barrena et al. (2011), sOUR profile corresponds to a low biodegradability substrate (typical bulking agent values). Low profiles are also linked with low biodegradable carbon quantities present in the substrate, despite presenting a chemical carbon composition superior to 40% (Table 4.1). However, according to Sánchez (2007) and as lignocellulosic material, available carbon in rice husk corresponds approximately to 30-40% of total carbon. No relevant visual differences between BB and TH conidia were observed at microscope level (from 40x to 100x augments). As shown

in Figure 4.6, BBs' growth and conidiation was easier to observe at eye level when compared to THs'. This difference has been maintained when working with RH as substrate at all reactor scales in this thesis.

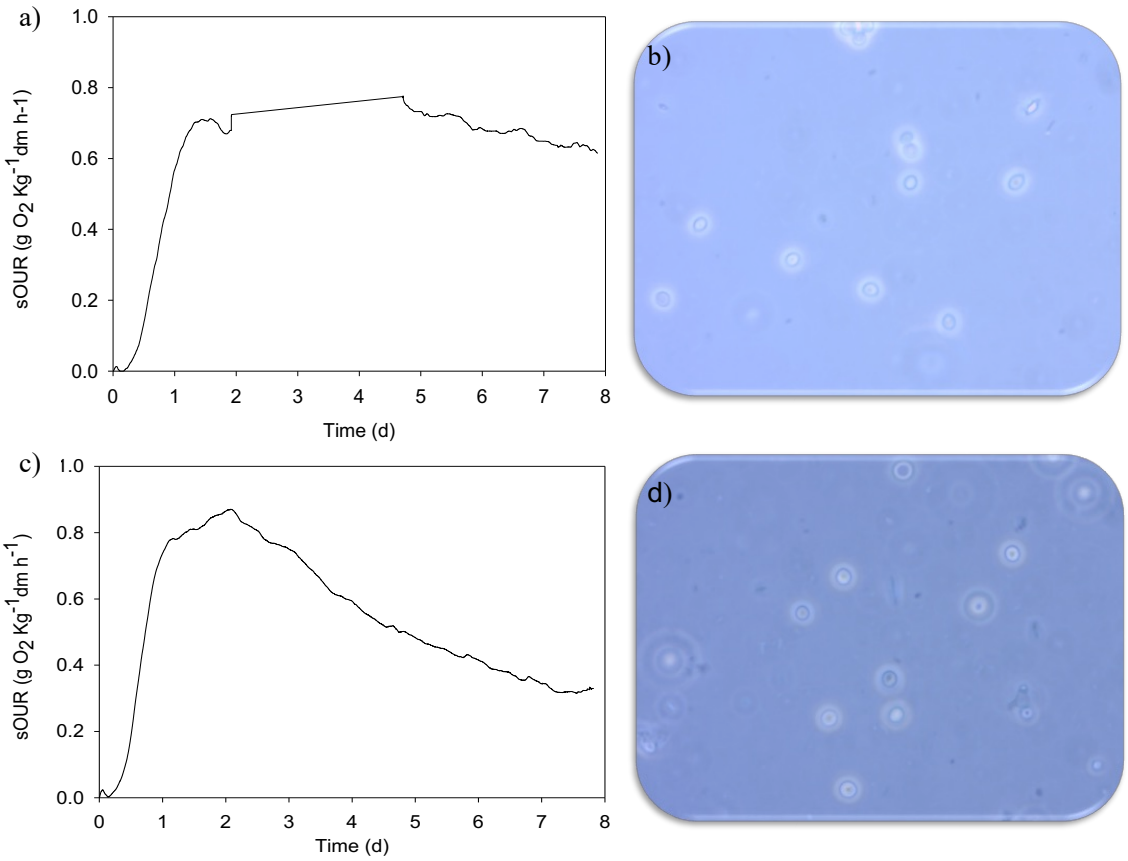



**Figure 4.5.** sOUR profiles and conidia visualization at 100x augments obtained in preliminary tests. a) and b): BB test; c) and d) TH test.

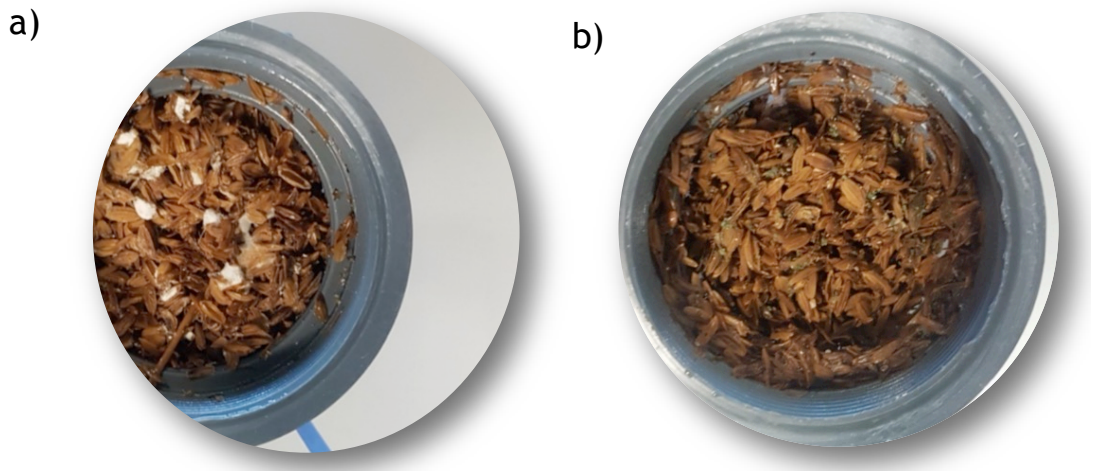



**Figure 4.6.** Example of reactor appearance for 0.5 L rice husk SSF. a) BB and b) TH.

Results in the preliminary tests confirmed the feasibility of using rice husk as a substrate for BB and TH conidia production. These tests also served as a starting point of the research, prior to process optimization tests.

### 4.4. 2. First time course tests

These tests aimed to determine process time for maximum conidia production as well as process parameters' evolution.

Figure 4.7 presents the first time course tests results for BB (a) and TH (b). When using BB, optimal production time in terms of conidia productivity were about 7.5-8 days (corresponding to 5.0 x $10^8$ conidia $g^{-1}$dm). Maximum productivity time was highly close to maximum conidia production time, stabilizing between days 8 and 11. Results were similar to the ones observed in preliminary tests, obtaining same optimal conidia production time. These values were consequent with typical 7-14 days' production cycle time in SSF fermentations, as stated by Jaronski and Mascarin (2017), being promising in terms of conidia productivity due to being closer to the lower half of their proposed time range. When using TH, optimal production time was 5.5-6 days (corresponding to 1.9x$10^9$ conidia $g^{-1}$dm). However, conidia production rapidly decreased and stabilized around 1.3x$10^9$ conidia $g^{-1}$dm. Maximum productivity and maximum conidia production were found at the same process time. Conidia production was higher than that obtained in the preliminary test, reaching superior order of magnitude in terms of both conidia production and productivity. According to various authors, optimal conidia production time when using *Trichoderma* spp. varies significantly, from 2-8 days (Verma et al., 2007) to 10-15 days (Srivastava et al., 2016) presenting huge differences between used substrates and strains.

In terms of sOUR, profiles were also very similar to those obtained in the preliminary tests, with similar maximums at similar times, between days 2.5-3 using BB and at 2-2.5 days using TH. Maximum sOUR time similarity between tests confirmed the low biodegradability potential of rice husk.

pH profiles were also similar between strains, showing decrease of approximately one unit during first 2-4 days. According to Krishna (2005), this might be caused by the production of organic acids, mostly citric and/or lactic. After that, pH followed an increase to values of 7.5 at the end of the experiment (day 12.5-13). As presented by Krishna (2005), optimal pH in SSF with filamentous fungi ranges between 3.8 and 6.

Dhar et al. (2016) established an optimal pH growth for BB of 6-7 at 25-30ºC, which coincides with the pH measured in this study before it started rising from day 6 onwards. Zhang and Yang (2015) presented an optimal pH for TH growth and conidiation of 6, with little decrease in conidia production between 6 and 7 and higher decreases in the rest of the tested range. This range corresponds to the measured pH in most of the TH first time course. According to the same author, possible inhibition might be applicable coinciding with the augment of the observed pH to values above 7 from day 6 onward, leading to conidia reduction and stabilization.

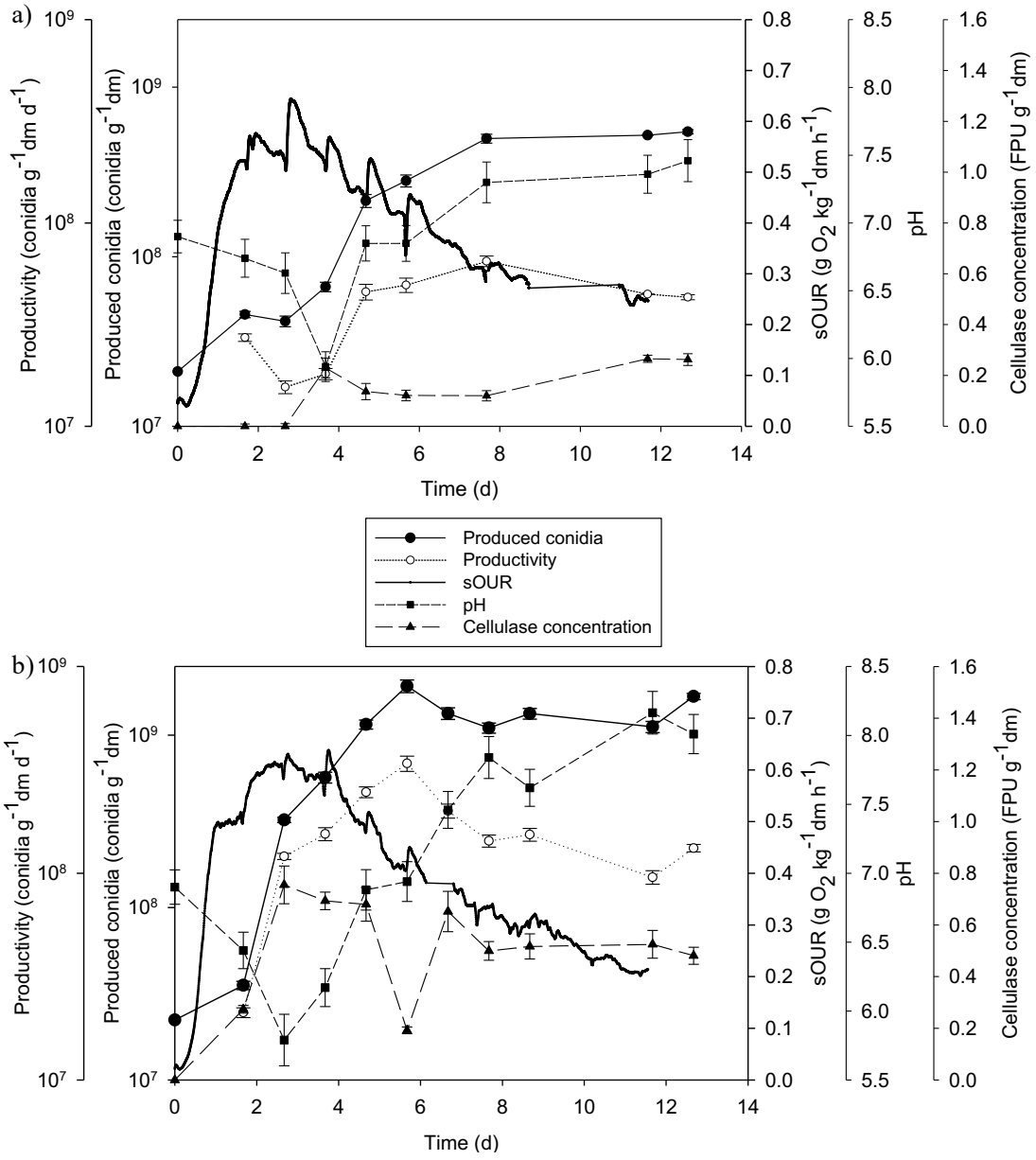



**Figure 4.7.** sOUR, conidia production, productivity, pH and cellulase concentration profiles obtained in first time course tests. sOUR curves were obtained using 6 reactors. a) BB test and b) TH test.

Cellulase profiles showed concentrations between 0.12–0.26 FPU $g^{-1}$dm using BB and of 0.2–0.75 FPU $g^{-1}$dm using TH, triplicating BB values at its maximum. However, enzymatic concentrations were very low when compared to those reported in specific cellulase production works by SSF using the same quantification method (Cerda, 2017; Marín, 2018). For TH, values were one order of magnitude lower than the concentration obtained by Lopez-Ramirez et al. (2018) also using TH. In addition, cellulase concentration remained very low in comparison to the previously mentioned works. Poor enzymatic productions coupled with low sOUR indicate low substrate biodegradability, probably due to presence of slow or non-biodegradable carbon in rice husk (Puyuelo et al., 2011).

### 4.4. 3. Mixing tests

These tests were performed to evaluate mixing effect on conidia production.

Figure 4.8 shows mixing tests results for BB (a) and TH (b). When using BB, increasing conidia production at increasing mixing times (40 h, 64 h and 88 h) was found, whereas the difference in maximum production (mixing at 88 h) resulted non-significant in comparison to conidia produced without mixing ($9.0x10^8$ conidia $g^{-1}$dm in the mixing reactor and $8.5x10^8$ conidia $g^{-1}$dm in the triplicate control, respectively). When using TH, results showed maximum conidia production in reactors with mixing times of 24 and 48h, each of them producing at least $9.0x10^8$ conidia $g^{-1}$dm, while reactors without mixing reached an average of $6.8x10^8$ conidia $g^{-1}$dm. Seemingly, mixing at 24 or 48 hours improved conidia production significantly. Obtained conidia were significantly less in comparison to time course test, in which TH reached productions of $1.9x10^9$ conidia $g^{-1}$dm (2.14 times higher) (Figure 4.5 b). However, maximum conidia production in time course test was only reached at the peak and was subsequently stabilised at around $1.3x10^9$ conidia $g^{-1}$dm (1.4 times higher), which could be explained due to using different rice husk supplies as substrates in the two tests (see Table 4.1).

It must be stated that mixing time could have changed optimal conidia production time, which could mean achieving higher conidia production if reactors had been kept fermenting for longer time periods. As presented by Krishna (2005), agitation is known to have adverse effects in SSF processes, particularly when using fungi, as it might disrupt fungal attachments and damage fungal mycelia, consequently increasing optimal conidia production time. This effect has been observed in BB mixing test. Fungi were not able to reattach properly, with the consequent loss in conidia production and productivity,

especially at both 40 and 64 hours of mixing. In comparison, mixing had a positive effect on TH, showing more robustness and adaptability, being able to not only reattach to the surface but also enhance its conidia production after mixing, also favouring TH fungal growth, as presented by Lopez-Ramirez et al. (2018). Possible productivity loss in comparison to no mixing strategy should be considered before making any decision on mixing application when working with TH.

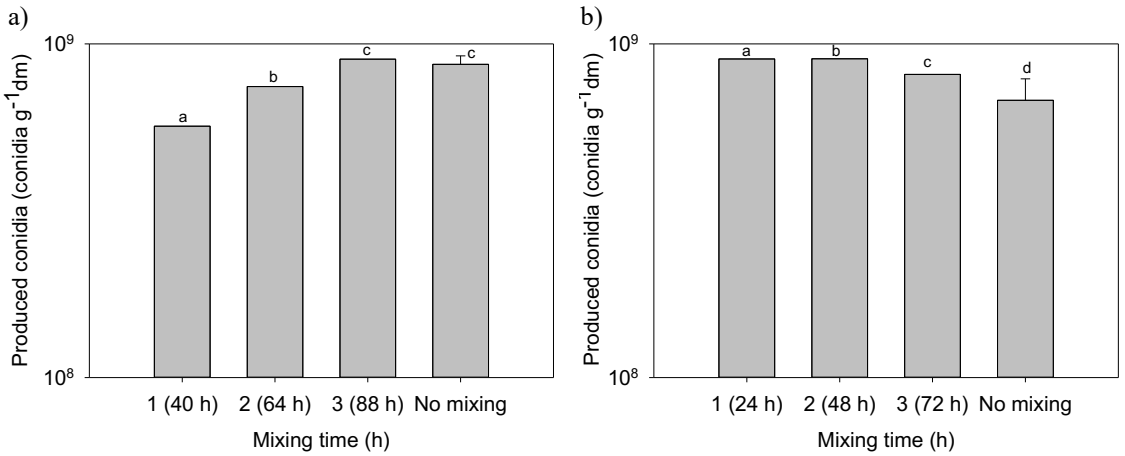



**Figure 4.8.** Conidia production in mixing tests. a) BB test and b) TH test.

### 4.4.4. DoEs

Two DoE sets were performed with each strain to maximize conidia productivity at the optimal time found in the previous time course tests. DoE 1 evaluated the effect of moisture, inoculum concentration and airflow, while DoE 2 evaluated temperature, C/N ratio and moisture.

Conidia productions of all DoE tests are shown in Figure 4.9. When using BB, in DoE 1, maximum production was achieved by reactor 6, topping at $1.9x10^9$ conidia $g^{-1}dm$, corresponding to initial moisture 65%, inoculum concentration of $5.5x10^6$ conidia $g^{-1}dm$ and airflow of 20 mL/min. In DoE2, maximum production was achieved by reactor 7, topping at $1.6x10^9$ conidia $g^{-1}dm$, corresponding to 25ºC temperature, 40 C/N ratio and 70% moisture. When using TH, in DoE 1, maximum production was achieved by reactors 13-14-15, topping at $1.2x10^9$ conidia $g^{-1}dm$, corresponding to the central parameter conditions, being initial moisture 55%, inoculum concentration $5.5x10^6$ conidia $g^{-1}dm$ and airflow of 40 mL/min. In DoE2, maximum production was achieved by reactor 3, topping at $2.0x10^9$ conidia $g^{-1}dm$, corresponding to 25ºC temperature, 55 C/N ratio and 60% moisture. Achieved BB conidia production was more than 2 times higher than the

best achieved production in time course 1. Achieved TH conidia production in DoE1 was similar to the one obtained after TH conidia production stabilization in time course 1, while in DoE2 it was as high as the obtained maximum in time course 1, being equal to the highest production obtained in BB DoE 1.

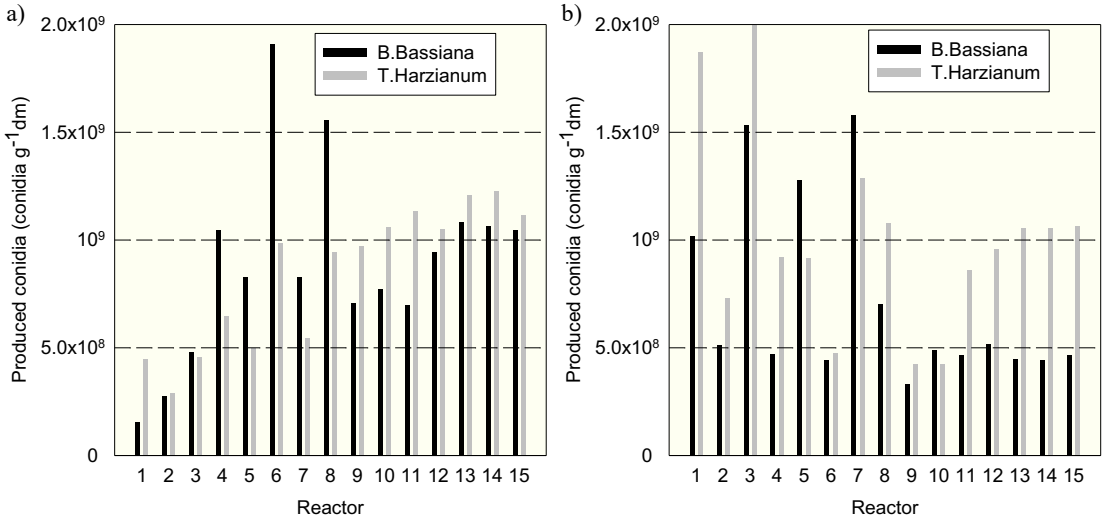



**Figure 4.9.** Conidia production in DoE tests. a) DoE1 and b) DoE2.

Statistical analysis of both DoE designs using a second-order polynomial approach is shown in Tables 4.5 (BB) and 4.6 (TH). All four models were significant in terms of p-value (<0.05), specially BB DoE2, which presented the minimal p-value possible using DesignExpert11. Both DoE1 presented significant lack of fit, which deemed them unusable to make predictions. However, BB DoE2 presented insignificant lack of fit, making the model valid for conidia production predictions. TH DoE2 lack of fit was not shown by the software, so it could not be used to interpret the results. As an alternative, the achieved $R^2$ value has been used as an approach to the significance of the model. Being of 0.89 in TH DoE2, indicates a more significant approach when compared to both DoE1 (in which $R^2$ values were of 0.74 and 0.71 respectively), albeit much less significant than BB DoE2, where $R^2$ was of 0.99, being the only design suitable for making conidia production predictions.

BB DoE1 analysis resulted in three significant parameters, being initial moisture (A), inoculum concentration (B) and inoculum concentration squared ($B^2$), while TH DoE1 analysis resulted in one significant parameter, being initial moisture squared ($A^2$). Even though inoculum concentration relevance differed between strains, airflow was deemed as insignificant for both. BB works dealing with similar configurations showed

higher effect of airflow on conidia production (Santa et al., 2005), even though the used substrate was a mixture of refused potatoes and sugarcane bagasse. This substrate possibly presented lower $AFP_R$ and biodegradability than rice husk, resulting in higher airflow needs due to difficulties in oxygen transfer. The same authors also analysed the effect of initial moisture, obtaining an optimal value of 65%, similar to the one obtained in this study. However, and as pointed by Manpreet et al. (2005), fungal strains can grow in a wide range of moistures varying from 40% to 80%, with optimal values being much more dependent on the used substrate than on the fungal strain. In comparison, the optimum moisture content was higher than the one obtained by Mishra et al. (2016) (51-54%), which might be due to configuration differences between the reactors used (Erlenmeyer flasks (Mishra et al., 2016) and PBBs (this thesis)). In comparison, no similar studies in terms of substrate and reactor configuration were found using TH or other *Trichoderma* species. The most similar TH optimization study was performed by Zhang and Yang (2015) using flasks as reactors and straw and wheat bran as substrates. That work reported an optimal moisture value of 75%, a higher value than the maximum values tested in DoE1. Airflow insignificance in both DoEs highlights the importance of substrate $AFP_R$ on oxygen transfer, which at this scale is more than sufficient for TH growth and conidiation when using rice husk as substrate.

BB DoE2 analysis resulted in most of the parameters (except for temperature x moisture (AC) and moisture squared ($C^2$)) as significant, while TH DoE2 analysis resulted in four significant parameters being temperature (A), moisture (C), temperature squared ($A^2$) and moisture squared ($C^2$). These results showed very high relevance for temperature (which was defined as the most important of all physical parameters affecting SSF performance by Krishna (2005)), being the most important parameter in terms of significance. DoE 2 also confirmed high relevance for moisture (with lower p-values than the ones obtained in previous DoE). C/N ratio was the least relevant parameter, even though it still was more relevant than airflow in DoE1.

In terms of temperature, several studies using different substrates have confirmed an optimal production temperature of 25 to 28ºC using different BB strains (Mishra et al., 2016; Pham et al., 2010; Dhar et al., 2016), corresponding to the one obtained in this test. Zhang and Yang (2015) found an optimal temperature of 30ºC, being 5ºC higher than the optimal value obtained (25ºC). Differences could be caused by the use of different TH strains. However, their conidia production heavily decreased from 32ºC onwards, as it

happened in the present study. In terms of moisture, observed differences in optimal values between DoEs might have been due to differences in the tested range.

**Table 4.5.** p-values on each BB DoE parameter, including lack of fit. p-values lower than 0.05 are considered significant.

| DoE 1 | p-value DoE1 | DoE 2 | p-value DoE2 |
|---|---|---|---|
| Model | 0.0438 | Model | < 0.0001 |
| A: $MC_I$(%) | 0.0296 | A: T (ºC) | < 0.0001 |
| B: IC (conidia·$g^{-1}$dm) | 0.0290 | B: C/N ratio | 0.0030 |
| C: AF (mL/min) | 0.9889 | C: $MC_I$ (%) | 0.0008 |
| AB | 0.7833 | AB | 0.0060 |
| AC | 0.7762 | AC | 0.0604 |
| BC | 0.7641 | BC | 0.0465 |
| $A^2$ | 0.1507 | $A^2$ | <0.0001 |
| $B^2$ | 0.0086 | $B^2$ | 0.0430 |
| $C^2$ | 0.0643 | $C^2$ | 0.1097 |
| Lack of fit | 0.0021 | Lack of fit | 0.1133 |
| $R^2$ | 0.7332 | $R^2$ | 0.9889 |

$MC_I$: initial moisture content; IC: inoculum concentration; AF: airflow; T: temperature

**Table 4.6.** p-values on each TH DoE parameter, including lack of fit. p-values lower than 0.05 are considered significant.

| DoE 1 | p-value DoE1 | DoE 2 | p-value DoE2 |
|---|---|---|---|
| Model | 0.0492 | Model | 0.0054 |
| A: $MC_I$ (%) | 0.1589 | A: T (ºC) | 0.0021 |
| B: IC (conidia $g^{-1}$dm) | 0.2866 | B: C/N ratio | 0.3884 |
| C: AF (mL/min) | 0.8098 | C: $MC_I$ (%) | 0.0017 |
| AB | 0.1708 | AB | 0.6247 |
| AC | 0.8052 | AC | 0.1867 |
| BC | 0.7602 | BC | 0.7419 |
| $A^2$ | 0.0030 | $A^2$ | 0.0227 |
| $B^2$ | 0.0697 | $B^2$ | 0.3233 |
| $C^2$ | 0.2230 | $C^2$ | 0.0026 |
| Lack of fit | 0.0298 | Lack of fit | - |
| $R^2$ | 0.7103 | $R^2$ | 0.8881 |

$MC_I$: initial moisture content; IC: inoculum concentration; AF: airflow; T: temperature

In relation to C/N ratio, production values superior to $10^9$ conidia $g^{-1}$dm were obtained with all tested C/N ratios. Theoretically, high C/N ratios are beneficial for fungal growth (Mishra et al., 2016; Sharma et al., 2002), however, nitrogen needs must also be taken into consideration as it will be less available for higher C/N ratios. Mishra et al. (2016) obtained maximum conidia production using BB with rice husk in comparison to other agricultural residues with a lower C/N ratio, with a value of 22.67. Carbon and nitrogen availability also plays a major role in fungal fermentations. When using rice husk, composition percentages between 40 and 50% correspond to carbon, while only 0.5 to 1.0% correspond to nitrogen depending on the rice husk supplied (Table 4.1). Carbon availability is not fully known by means of chemical C/N analysis. Thus, biodegradable C/N ratio is needed for a better knowledge on available carbon. It might also be possible that the added N solution (ammonium sulphate) was not sufficient to satisfy culture needs. This behaviour is consistent with the few observed differences in terms of produced conidia between different C/N ratios, as it is also necessary for fungal growth (Dhar et al., 2016).

Optimal conditions found with BB DoE 1 are shown in the form of 3D surface plot in Figure 4.10, while optimal conditions using BB DoE2 are shown in Figure 4.11. Using DoE1 equation ($+4.29770 +0.158560A +1.71190x10^{-7}B -0.031302C +4.69538x10^{-10}AB -0.000109AC +2.56439x10^{-10}BC -0.001285A^2 -1.56903x10^{-14}B^2 +0.000448C^2$) ($R^2$=0.7332) optimal production values were initial moisture 62-62.5%, inoculum concentration 6.5-6.6x$10^6$ conidia $g^{-1}$dm and airflow 20 mL/min. Using DoE2 equation ($+15.01230 -0.386005A +0.042742B -0.026841C -0.000504AB +0.000399AC +0.000203BC +0.005518A^2 -0.000144B^2 +0.000234C^2$) ($R^2$=0.9889) optimal production values were temperature 25ºC, C/N ratio 55 and initial moisture 70%. Optimal moisture value was a little higher when compared to DoE 1 value. However, the studied range was slightly different between tests, which might have displaced the optimum to higher values.

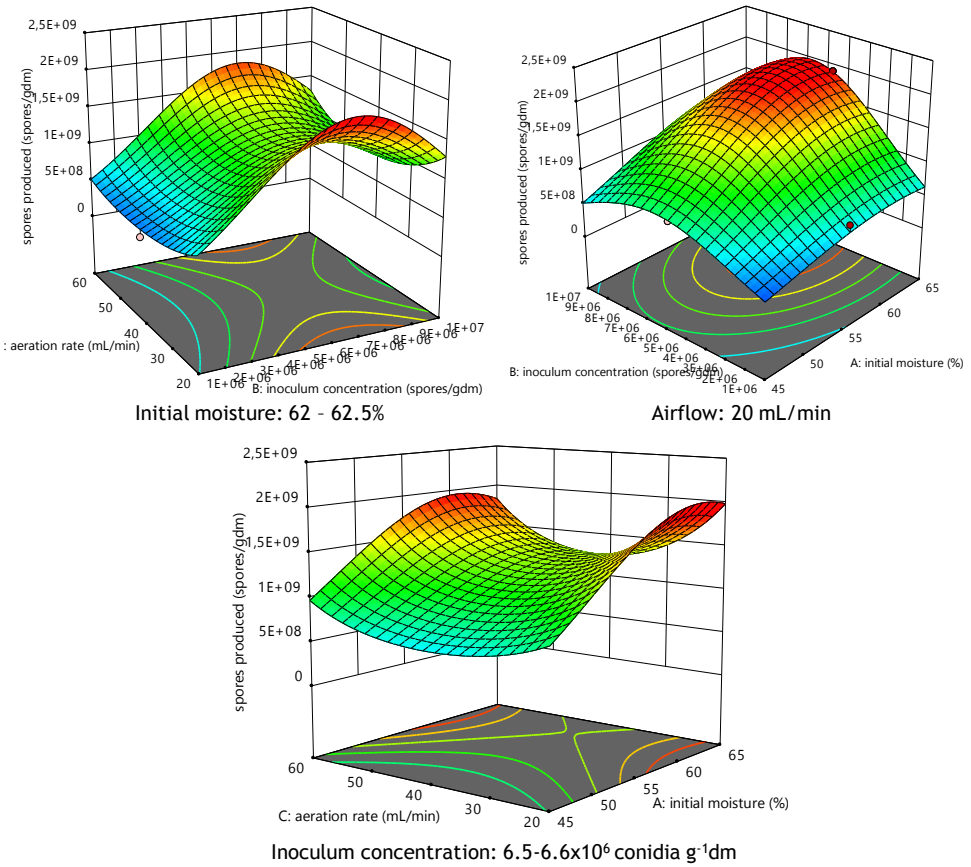



**Figure 4.10.** 3D surface plots obtained for optimal parameter values in BB DoE1 test.

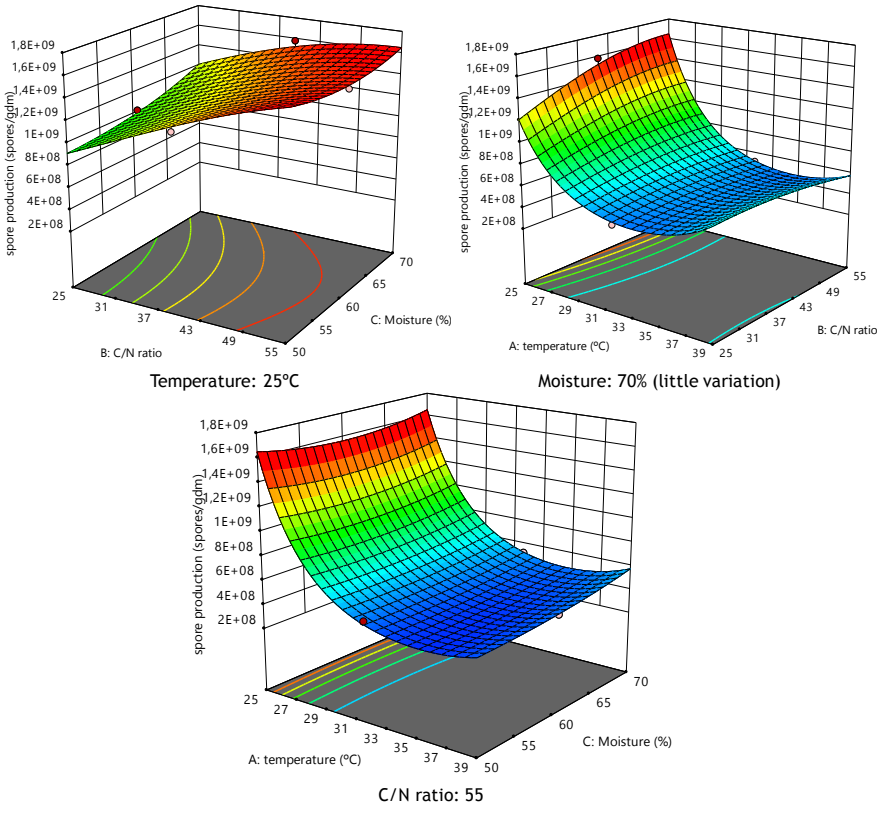



**Figure 4.11.** 3D surface plots obtained for optimal parameter values in BB DoE2 test.

Optimal conditions with TH DoE1 are shown in the form of 3D surface plot in figure 4.12, while optimal conditions using TH DoE2 are shown in Figure 4.13. Using DoE1 equation (+0.070511 +0.328833A -1.72618x10$^{-8}$B -0.010144C +1.90317x10$^{-9}$AB -0.000070AC -1.91895x10$^{-10}$BC -0.003002A$^2$ -6.33619x10$^{-15}$B$^2$ +0.000194C$^2$) ($R^2$=0.7103) optimal production values were initial moisture 56-56.5%, inoculum concentration 6.5-6.6x10$^6$ conidia g$^{-1}$dm and airflow 20 or 60 mL/min. Using DoE2 equation (+5.67152 -0.219148A +0.005105B +0.220239C +0.000167AB +0.000735AC +0.000078BC +0.002322A$^2$ -0.000170B$^2$ -0.001936C$^2$) ($R^2$=0.8881) optimal production values were temperature 25ºC, C/N ratio 40 and initial moisture 62%. However, none of the TH models can be used to obtain reliable conidia productions predictions, even though DoE2 model presented way higher significance than DoE1.

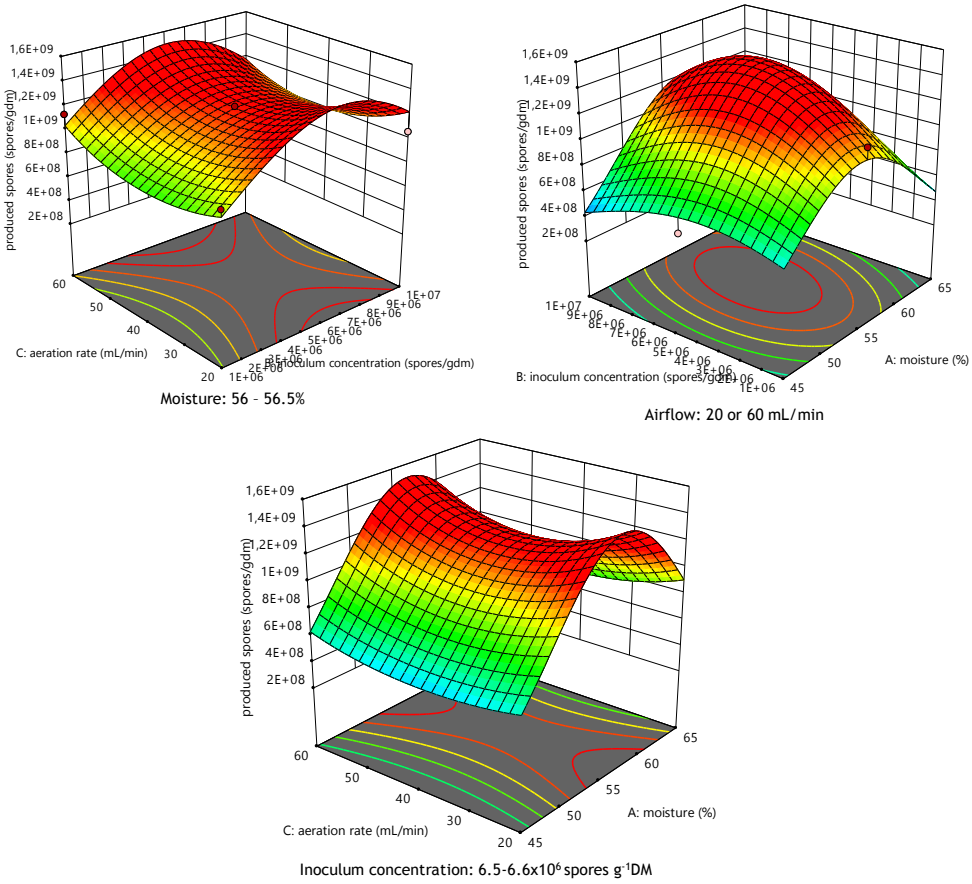



**Figure 4.12.** 3D surface plots obtained for optimal parameter values in TH DoE1 test.

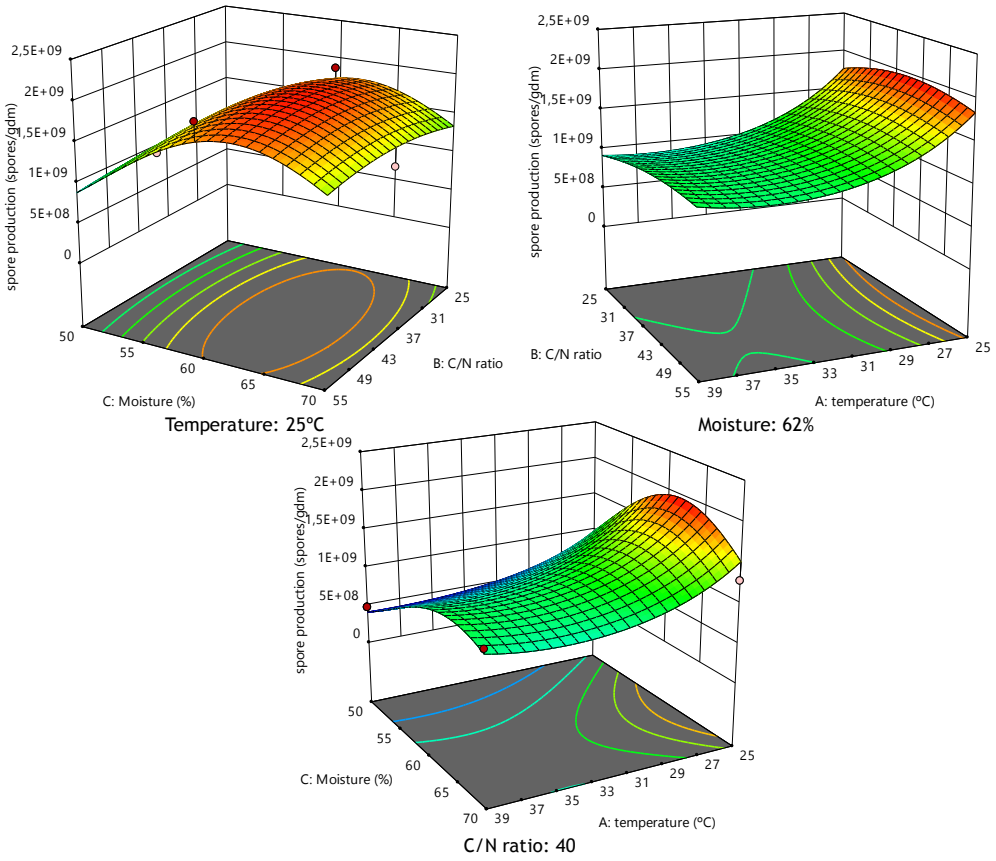



**Figure 4.13.** 3D surface plots obtained for optimal parameter values in TH DoE2 test.

### 4.4. 5. Second time course test

These tests aimed to validate the obtained conditions in previous tests.

Figure 4.14 presents second time course tests results for BB (a) and TH (b). When using BB, optimal conidia production time in terms of productivity was about 7.5-8 days (corresponding to $9.0x10^8$ conidia $g^{-1}$dm). Between days 12 and 18, BB conidia production value was stabilized around $6.0x10^8$ conidia $g^{-1}$dm. When using TH, maximum conidia production nearly doubled the one observed in time course 1. The optimal production time in terms of conidia productivity was about 5.5-6 days (corresponding to $1.4x10^9$ conidia $g^{-1}$dm). Having reached its peak, conidia production decreased a little before stabilizing at nearly the same value as in maximum production between days 9 and 12.

Despite using optimal conditions found in previous tests and nearly doubling time course 1 results, obtained conidia production was half of the maximum obtained in the DoEs with BB and one quarter lower than the maximum obtained in both time course 1 and DoE2 with TH.

These differences might have been caused by the use of different rice husk supplies. Rice husk supply used in the current tests presented lower biodegradability potential in comparison to the one used in time course 1, which was confirmed by means of sOUR values: while maximum sOUR achieved in previous time course tests was at least of 0.64 $gO_2\ kg^{-1}dm\ h^{-1}$ for both strains, in this time course only maximum values of 0.37 $gO_2\ kg^{-1}dm\ h^{-1}$ (BB) and 0.44 $gO_2\ kg^{-1}dm\ h^{-1}$ (TH) were reached., showing even lower biodegradability despite using more suitable conditions for conidia production. These oxygen level values correspond to the minimums observed for both strains in this Chapter. C/N could be another parameter which might have reduced conidia production, particularly due to nitrogen content, which was even less present in comparison to other tests (0.42% in this time course test vs 0.52% in the first time course and even higher differences if compared to the rice husk supplies used in the DoEs).

Presence of non-inoculated *Aspergillus Niger* (AN) was detected during BB fermentation. Figure 4.15 shows profiles (4.15 a) and photos of BB and AN conidia at 100x augments (4.15 b) and contaminated culture plate growth (4.15 c), effectively showing a mixed culture. AN is a common contaminant in rice and its by-products, (Streit et al., 2012; Aydin et al., 2011; Fredlund et al., 2009). AN conidia were also confirmed as capable of withstanding autoclaving. AN production was observed in BB tests from day 5 onwards, rising to approximate values close to $1x10^8$ conidia $g^{-1}dm$ (approximately 10% of the maximum BB conidia production). To the authors' knowledge, no BB strain presenting antifungal properties against AN have been reported. Consequently, it is possible for AN to take advantage of its growth, resulting in contaminated cultures. Despite the contamination, it must be stated that BB is still present, approximately at about 90% of the total conidia production, at least at microscopical level. Differences between BB and AN conidia can be visually observed using both 40x and 100x augments (Figure 4.15 a). In contrast, no contamination was observed when using TH, not in time course 2 nor in any other TH fermentation. AN conidia were unable to growth in a culture with TH due to its antagonistic properties (Verma et al., 2007).

In both fermentations, initial pH was close to 6-6.5 and ended near 8.0. These profiles differ from those in the first time course tests, where pH decreased between days 3-5 before rising to values close to 8.0 (figure 4.7). As shown in previous time course test, conidia production stabilization is observed after pH has risen to values above 7, corresponding to day 7 in BB and to day 5 in TH.

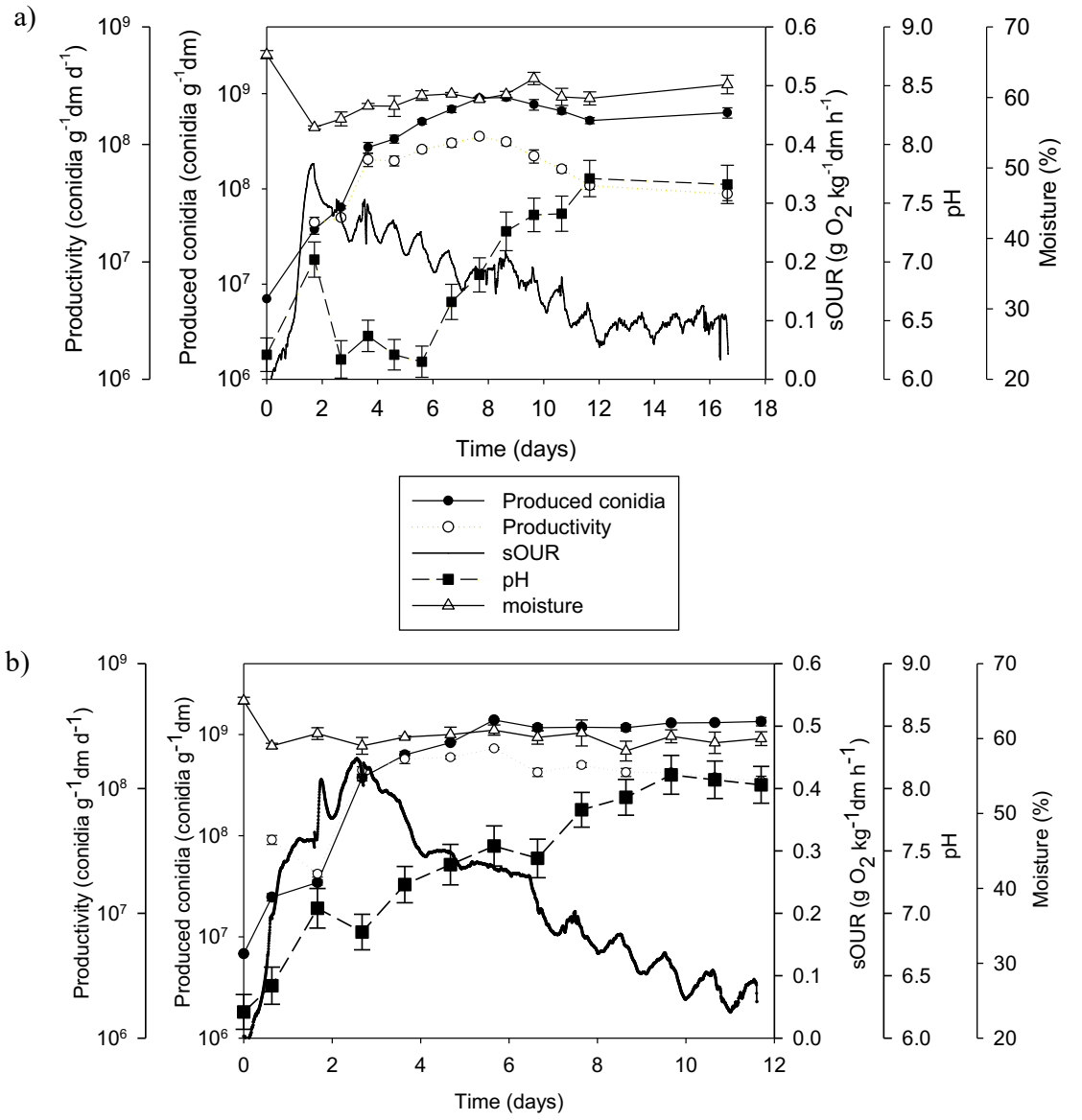



**Figure 4.14.** sOUR, produced conidia, productivity, pH and moisture profiles obtained in first time course tests. sOUR curves were obtained using 12 reactors. a) BB test and b) TH test.

Moisture content profile showed maintenance of optimal values during the whole test with both strains, with stable values around 60%. Even though initial moisture content has been optimized for several substrates and despite its relevance in fungal SSF (Krishna, 2005), to our knowledge, moisture evolution during BB or TH SSF process had not been presented in any work previous to this test.

Cellulase analysis was not significant for both strains. Cellulase production was very low and nearly undetectable, with values ranging from 0 to 0.2 FPU $g^{-1}$dm at their maximum. These values were similar to the maximum achieved in BB time course 1, albeit inferior to the ones for TH, where they were at least measurable. TH differences

might be due to the use of a substrate with very low biodegradability in comparison to the previous time course substrate. Similarly, Sánchez-Corzo et al. (2021) did not obtain cellulase production using TH when testing various lignocellulolytic enzymes production using various wood rot fungi. Nevertheless, the method used to analyse cellulase concentration seemed unreliable for rice husk samples, as values were too low to be correctly quantified. Moreover, no other works on the production of cellulases using BB strains have been found, as BB is mainly used as entomopathogen but not as enzyme producer (Jaronski and Mascarin, 2017).

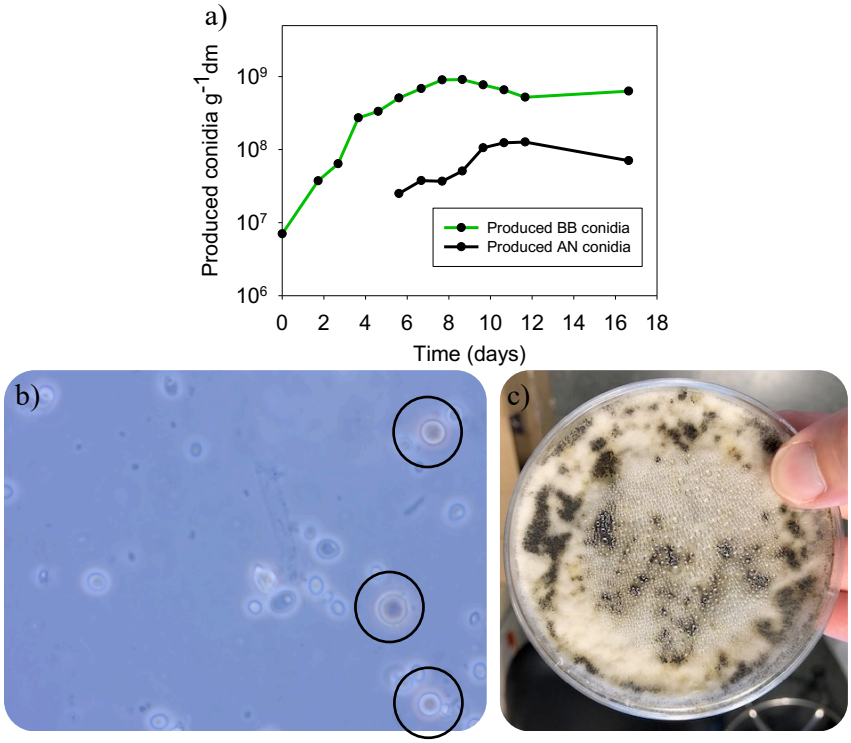



**Figure 4.15.** Mixture culture of BB contaminated with AN. a) Produced conidia, b) Conidia appearance at 100x augments (AN conidia are highlighted over BB conidia) and c) Plate growth of the extract obtained at the maximum conidia production day.

## 4.5. Global analysis

Figure 4.16 shows conidia production obtained in all tests and its distribution using box plots. Figure 4.16 a) shows the general distribution of all spore productions obtained in all tests, which is asymmetrical for both strains. With BB, most of conidia productions range between $5.0x10^8$ and $1.0x10^9$ conidia $g^{-1}$dm, while with TH range between $6.0x10^8$ and $1.2x10^9$ conidia $g^{-1}$dm. However, in BB plot median value is located at $7.0x10^8$ conidia $g^{-1}$dm while in TH it is located at $1.0x10^9$ conidia $g^{-1}$dm, showing

higher productions in TH 3rd quartile. BB presents a widest liable production range (between $4.0x10^8$ and $1.6x10^9$ conidia $g^{-1}$dm), which includes Mishra et al. (2016) BB conidia production obtained with rice husk (the only comparable BB production found in the literature using this substrate). TH productions are more consistent, showing higher robustness due to a lower reliable range (from $4.5x10^8$ to $1.3x10^9$ conidia $g^{-1}$dm). Few outliers are present with both strains, showing the robustness of the data.

Figure 4.16 b) shows distributions in all BB tests, whereas figure 4.16 c) shows it with TH. Tested parameters in DoE1 (initial moisture, inoculum concentration and airflow) had a higher impact on BB conidia production, while tested parameters in DoE2 (temperature, C/N ratio and initial moisture) had a higher impact on TH conidia production, as it can be seen both with obtained ranges and media values. Second time course productions were in the 3rd quartile in BB and in the 4th quartile in TH, demonstrating the feasibility of our defined optimal conditions. As higher productions were reached with both strains when comparing to time course 1. Nevertheless, they were not the maximum reached productions due to using low biodegradability substrate with little nitrogen. Using of above optimal C/N ratios might have reduced conidia production, as presented by Mishra et al. (2016).

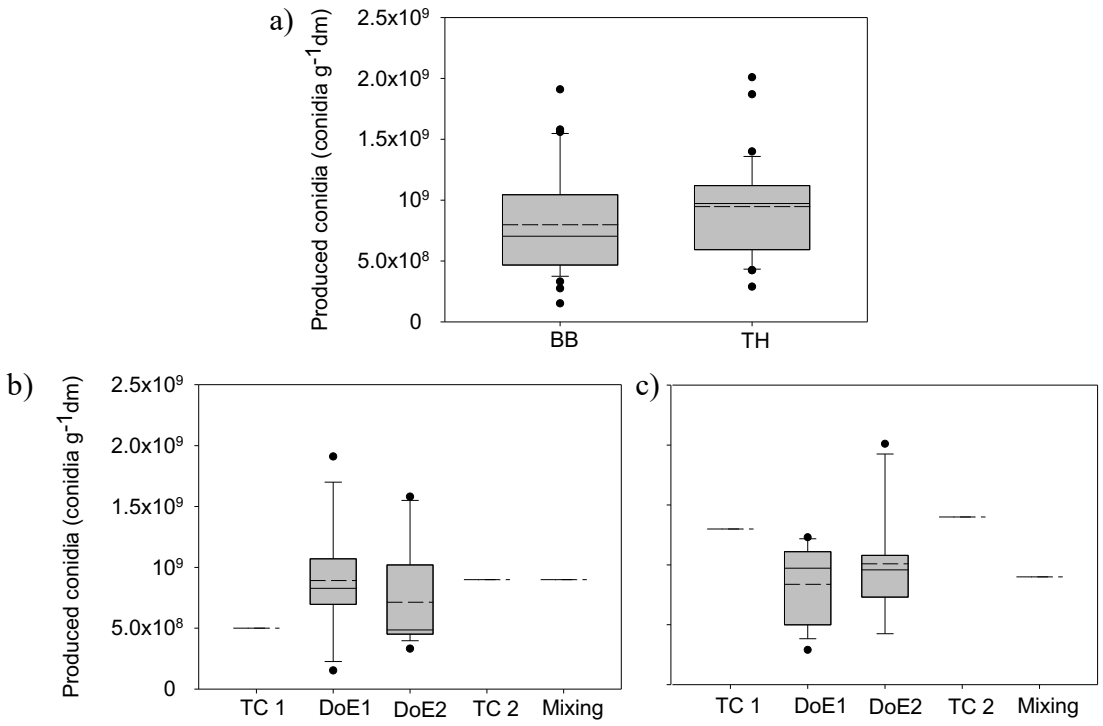



**Figure 4.16.** Conidia production boxplots. a) all fermentations, b) BB fermentations) and c) TH fermentations. Mean values are presented with dashed lines.

These results allow us to determine a highly probable conidia production range (between $5.0x10^8$ and $1.3x10^9$ conidia $g^{-1}$dm) which is independent of the used rice husk supply. This behaviour demonstrates the robustness of the process and the scale-up feasibility of the chosen substrate. It also must be stated that BB conidia production has been affected by the growth of contaminant AN (a common contaminant in several food and food wastes as presented by Gil-Serna et al. (2019)). As a result, production was lower in certain batches, meaning that obtained counts might have been higher after a more suitable substrate pretreatment. However, AN's conidia resistance to autoclaving cycles must also be taken into consideration.

With all these data, it is possible to define conidia production values for all analysed conditions, with certainty that conidia production using rice husk will be in the previously defined interval if the parameters are kept within range. These values are: initial moisture 55-70%, inoculum concentration $5.5x10^6$–$2.1x10^7$ conidia $g^{-1}$dm, airflow 20–60 mL $min^{-1}$, temperature 25ºC and C/N ratio 25–95.

Summarized results of conidia production and parameter conditions in all tests are presented in Table 4.7, whereas summarized results of conidia production, productivity and conidia quotients are presented in Table 4.8. As it has been stated, all parameters which presented relevance on conidia production (even if it was minimal) have been successfully optimized in a correspondent reduction range or even to a certain value. The only exception is C/N ratio, as highest conidia productions have been achieved with various C/N values ranging from 25 in DoEs to 79-95 in time course 1 and time course 2. Using BB, highest conidia production was achieved using rice husk 2 (corresponding to a C/N ratio of 56.0). Using TH, maximum was achieved using rice husk 3 (corresponding to a C/N ratio of 53.6), even though maximum conidia production was similar to the one obtained with rice husk 1 (corresponding to a C/N ratio of 78.8). These results suggest that further tests should be proposed to find lower optimal C/N values, which would be more consistent with other similar studies using the same substrate (Mishra et al., 2016). At the same time, it is advisable to use the same or similar rice husk as substrate when performing optimization or comparative tests to reduce differences caused by the use of different rice husk supplies.

**Table 4.7.** Summary of the results obtained in all BB and TH optimization tests (produced conidia and initial parameters).

| Test | Produced BB conidia (conidia $g^{-1}$dm) | Produced TH conidia (conidia $g^{-1}$dm) | MC (%) | IC (conidia $g^{-1}$dm) | AF (mL $min^{-1}$) | T (ºC) | C/N ratio |
|---|---|---|---|---|---|---|---|
| **Preliminary test** | $7.0x10^8$ | $8.4x10^8$ | 67.5 | $2.1x10^7$ | 20 | 25 | 79 |
| **Time course** | $5.0x10^8$ | $1.9x10^9$ | 63.5 | $2.1x10^7$ | 20 | 25 | 79 |
| **Mixing** | $9.0x10^8$ | $9.0x10^8$ | 64 | $1.0x10^7$ | 20 | 25 | 56 |
| **DoE1 R1** | $1.5x10^8$ | $4.5x10^8$ | 45 | $1.0x10^6$ | 40 | 25 | 56 |
| **DoE1 R2** | $2.8x10^8$ | $2.9x10^8$ | 65 | $1.0x10^6$ | 40 | 25 | 56 |
| **DoE1 R3** | $4.8x10^8$ | $4.5x10^8$ | 45 | $1.0x10^7$ | 40 | 25 | 56 |
| **DoE1 R4** | $1.1x10^9$ | $6.5x10^8$ | 65 | $1.0x10^7$ | 40 | 25 | 56 |
| **DoE1 R5** | $8.3x10^8$ | $5.0x10^8$ | 45 | $5.5x10^6$ | 20 | 25 | 56 |
| ***DoE1 R6** | $1.9x10^9$ | $9.9x10^8$ | 65 | $5.5x10^6$ | 20 | 25 | 56 |
| **DoE1 R7** | $8.3x10^8$ | $5.4x10^8$ | 45 | $5.5x10^6$ | 60 | 25 | 56 |
| **DoE1 R8** | $1.6x10^9$ | $9.4x10^8$ | 65 | $5.5x10^6$ | 60 | 25 | 56 |
| **DoE1 R9** | $7.0x10^8$ | $9.7x10^8$ | 55 | $1.0x10^6$ | 20 | 25 | 56 |
| **DoE1 R10** | $7.7x10^8$ | $1.1x10^9$ | 55 | $1.0x10^7$ | 20 | 25 | 56 |
| **DoE1 R11** | $7.0x10^8$ | $1.1x10^9$ | 55 | $1.0x10^6$ | 60 | 25 | 56 |
| **DoE1 R12** | $9.4x10^8$ | $1.1x10^9$ | 55 | $1.0x10^7$ | 60 | 25 | 56 |
| ****DoE1 R13** | $1.1x10^9$ | $1.2x10^9$ | 55 | $5.5x10^6$ | 40 | 25 | 56 |
| ****DoE1 R14** | $1.1x10^9$ | $1.2x10^9$ | 55 | $5.5x10^6$ | 40 | 25 | 56 |
| ****DoE1 R15** | $1.0x10^9$ | $1.1x10^9$ | 55 | $5.5x10^6$ | 40 | 25 | 56 |

**Table 4.7. (cont).** Summary of the results obtained in all BB and TH optimization tests (produced conidia and initial parameters).

| Test | Produced BB conidia (conidia $g^{-1}$dm) | Produced TH conidia (conidia $g^{-1}$dm) | MC (%) | IC (conidia $g^{-1}$dm) | AF (mL $min^{-1}$) | T (ºC) | C/N ratio |
|---|---|---|---|---|---|---|---|
| **DoE2 R1** | $1.0x10^9$ | $1.9x10^9$ | 60 | $7.5x10^6$ | 20 | 25 | 25 |
| **DoE2 R2** | $5.1x10^8$ | $7.3x10^8$ | 60 | $7.5x10^6$ | 20 | 39 | 25 |
| ****DoE2 R3** | $1.5x10^9$ | $2.0x10^9$ | 60 | $7.5x10^6$ | 20 | 25 | 55 |
| **DoE2 R4** | $4.7x10^8$ | $9.2x10^8$ | 60 | $7.5x10^6$ | 20 | 39 | 55 |
| **DoE2 R5** | $1.3x10^9$ | $9.1x10^8$ | 50 | $7.5x10^6$ | 20 | 25 | 40 |
| **DoE2 R6** | $4.4x10^8$ | $4.8x10^8$ | 50 | $7.5x10^6$ | 20 | 39 | 40 |
| ***DoE2 R7** | $1.6x10^9$ | $1.3x10^9$ | 70 | $7.5x10^6$ | 20 | 25 | 40 |
| **DoE2 R8** | $7.0x10^8$ | $1.1x10^9$ | 70 | $7.5x10^6$ | 20 | 39 | 40 |
| **DoE2 R9** | $3.3x10^8$ | $4.3x10^8$ | 50 | $7.5x10^6$ | 20 | 32 | 25 |
| **DoE2 R10** | $4.9x10^8$ | $4.3x10^8$ | 50 | $7.5x10^6$ | 20 | 32 | 55 |
| **DoE2 R11** | $4.7x10^8$ | $8.6x10^8$ | 70 | $7.5x10^6$ | 20 | 32 | 25 |
| **DoE2 R12** | $5.1x10^8$ | $9.6x10^8$ | 70 | $7.5x10^6$ | 20 | 32 | 55 |
| **DoE2 R13** | $4.5x10^8$ | $1.1x10^9$ | 60 | $7.5x10^6$ | 20 | 32 | 40 |
| **DoE2 R14** | $4.4x10^8$ | $1.1x10^9$ | 60 | $7.5x10^6$ | 20 | 32 | 40 |
| **DoE2 R15** | $4.6x10^8$ | $1.1x10^9$ | 60 | $7.5x10^6$ | 20 | 32 | 40 |
| **2nd Time course** | $9.0x10^8$ | $1.4x10^9$ | 65 | $6.8x10^6$ | 20 | 25 | 95 |

MC: moisture content; IC: inoculum concentration; AF: airflow; T: temperature; *Best DoE conditions for BB; **Best DoE conditions for TH. Results in mixing rows correspond to the best mixing conidia production reactor.

**Table 4.8.** Summary of the results obtained in all BB and TH optimization tests (produced conidia, productivities and conidia quotients).

| Test | Produced BB conidia (conidia $g^{-1}dm$) | Produced TH conidia (conidia $g^{-1}dm$) | Productivity in BB tests (conidia $g^{-1}dm\ d^{-1}$) | Productivity in TH tests (conidia $g^{-1}dm\ d^{-1}$) | BB CQ | TH CQ |
|---|---|---|---|---|---|---|
| **Preliminary test** | $7.0x10^8$ | $8.4x10^8$ | $8.9x10^7$ | $1.1x10^8$ | 34 | 40 |
| **Time course** | $5.0x10^8$ | $1.9x10^9$ | $6.5x10^7$ | $3.4x10^8$ | 24 | 86 |
| **Mixing** | $9.0x10^8$ | $9.0x10^8$ | $1.1x10^8$ | $1.3x10^8$ | 90 | 90 |
| **DoE1 R1** | $1.5x10^8$ | $4.5x10^8$ | $2.0x10^7$ | $7.8x10^7$ | 150 | 450 |
| **DoE1 R2** | $2.8x10^8$ | $2.9x10^8$ | $3.6x10^7$ | $5.1x10^7$ | 280 | 290 |
| **DoE1 R3** | $4.8x10^8$ | $4.5x10^8$ | $6.2x10^7$ | $8.0x10^7$ | 48 | 45 |
| **DoE1 R4** | $1.1x10^9$ | $6.5x10^8$ | $1.4x10^8$ | $1.1x10^8$ | 110 | 65 |
| **DoE1 R5** | $8.3x10^8$ | $5.0x10^8$ | $1.1x10^8$ | $8.8x10^7$ | 151 | 96 |
| ***DoE1 R6** | $1.9x10^9$ | $9.9x10^8$ | $2.5x10^8$ | $1.7x10^8$ | 346 | 180 |
| **DoE1 R7** | $8.3x10^8$ | $5.4x10^8$ | $1.1x10^8$ | $9.5x10^7$ | 151 | 98 |
| **DoE1 R8** | $1.6x10^9$ | $9.4x10^8$ | $2.0x10^8$ | $1.7x10^8$ | 291 | 171 |
| **DoE1 R9** | $7.0x10^8$ | $9.7x10^8$ | $9.2x10^7$ | $1.7x10^8$ | 700 | 970 |
| **DoE1 R10** | $7.7x10^8$ | $1.1x10^9$ | $1.0x10^8$ | $1.9x10^8$ | 77 | 111 |
| **DoE1 R11** | $7.0x10^8$ | $1.1x10^9$ | $9.1x10^7$ | $2.0x10^8$ | 700 | 1110 |
| **DoE1 R12** | $9.4x10^8$ | $1.1x10^9$ | $1.2x10^8$ | $1.8x10^8$ | 94 | 110 |
| ****DoE1 R13** | $1.1x10^9$ | $1.2x10^9$ | $1.4x10^8$ | $2.1x10^8$ | 200 | 218 |
| ****DoE1 R14** | $1.1x10^9$ | $1.2x10^9$ | $1.4x10^8$ | $2.2x10^8$ | 200 | 218 |
| ****DoE1 R15** | $1.0x10^9$ | $1.1x10^9$ | $1.4x10^8$ | $2.0x10^8$ | 182 | 200 |

**Table 4.8. (cont)** Summary of the results obtained in all BB and TH optimization tests (produced conidia, productivities and conidia quotients).

| Test | Produced BB conidia (conidia $g^{-1}dm$) | Produced TH conidia (conidia $g^{-1}dm$) | Productivity in BB tests (conidia $g^{-1}dm\ d^{-1}$) | Productivity in TH tests (conidia $g^{-1}dm\ d^{-1}$) | BB CQ | TH CQ |
|---|---|---|---|---|---|---|
| **DoE2 R1** | $1.0x10^9$ | $1.9x10^9$ | $1.3x10^8$ | $3.3x10^8$ | 133 | 253 |
| **DoE2 R2** | $5.1x10^8$ | $7.3x10^8$ | $6.7x10^7$ | $1.3x10^8$ | 68 | 97 |
| ****DoE2 R3** | $1.5x10^9$ | $2.0x10^9$ | $2.0x10^8$ | $3.6x10^8$ | 200 | 267 |
| **DoE2 R4** | $4.7x10^8$ | $9.2x10^8$ | $6.2x10^7$ | $1.6x10^8$ | 63 | 123 |
| **DoE2 R5** | $1.3x10^9$ | $9.1x10^8$ | $1.7x10^8$ | $1.6x10^8$ | 173 | 121 |
| **DoE2 R6** | $4.4x10^8$ | $4.8x10^8$ | $5.8x10^7$ | $8.4x10^7$ | 59 | 64 |
| ***DoE2 R7** | $1.6x10^9$ | $1.3x10^9$ | $2.1x10^8$ | $2.3x10^8$ | 213 | 173 |
| **DoE2 R8** | $7.0x10^8$ | $1.1x10^9$ | $9.2x10^7$ | $1.9x10^8$ | 93 | 148 |
| **DoE2 R9** | $3.3x10^8$ | $4.3x10^8$ | $4.3x10^7$ | $7.5x10^7$ | 44 | 57 |
| **DoE2 R10** | $4.9x10^8$ | $4.3x10^8$ | $6.4x10^7$ | $7.5x10^7$ | 65 | 57 |
| **DoE2 R11** | $4.7x10^8$ | $8.6x10^8$ | $6.1x10^7$ | $1.5x10^8$ | 63 | 115 |
| **DoE2 R12** | $5.1x10^8$ | $9.6x10^8$ | $6.8x10^7$ | $1.7x10^8$ | 68 | 128 |
| **DoE2 R13** | $4.5x10^8$ | $1.1x10^9$ | $5.9x10^7$ | $1.9x10^8$ | 60 | 148 |
| **DoE2 R14** | $4.4x10^8$ | $1.1x10^9$ | $5.8x10^7$ | $1.9x10^8$ | 59 | 148 |
| **DoE2 R15** | $4.6x10^8$ | $1.1x10^9$ | $6.1x10^7$ | $1.9x10^8$ | 61 | 148 |
| **2nd Time course** | $9.0x10^8$ | $1.4x10^9$ | $1.2x10^8$ | $2.1x10^8$ | 128.6 | 211.8 |

CQ: conidia quotient; *Best DoE conditions for BB; **Best DoE conditions for TH. Results in mixing rows correspond to the best mixing conidia production reactor

To have a global view of the respiration indexes obtained in most tests, Table 4.9 shows achieved maximum sOUR, time in which maximum sOUR was achieved and COC for all tests. The exception are the DoEs, showing only those that reached highest conidia production on each design. Very low biodegradability has been observed in most of the tests, with maximum sOUR being of 1.55 g $O_2$ $kg^{-1}$dm $h^{-1}$ for BB (DoE1) and 0.94 g $O_2$ $kg^{-1}$dm $h^{-1}$ for TH (mixing). Despite being way higher than most of the obtained values, they remain in the low biodegradability spectrum according to Barrena et al. (2011). Even though sOUR differences are important between substrate batches and between different conditions, the potential biodegradability of the substrate has remained at very low values in all tests. For TH, maximum sOUR values have not been obtained in maximum conidia production reactors. In fact, huge differences in respiration indexes between similar conidia productions have been observed with both strains, with special mention to both maximums DoEs. No correlation between sOUR and conidia production when working with BB or TH and rice husk as substrate has been found.

Previous works to this thesis had presented correlations between fungal growth and conidia production (Santa et al., 2005; Lopez-Ramirez et al., 2018) using both BB or TH and other substrates. In this work, fungal development varied significantly between rice husk supplies, being able to produce conidia at similar quantities and times but with significantly different mycelial growth. More tests comparing substrates presenting different fungal growth should be performed before reaching a conclusion on correlation between fungal growth and conidia production.

Despite the inexistent correlation with conidia production, sOUR determination remains as completely necessary to monitor fungal SSF process. It would be impossible to correctly monitor fungal growth without the correspondent on-line sOUR profile (Puyuelo et al., 2010).

**Table 4.9. s**OUR, COC, days of maximum respiration and conidia production in all tests. Data shown for DoEs corresponds to the best performing reactors in terms of maximum conidia production.

| | **sOUR max ($g\ O_2\ kg^{-1}dm\ h^{-1}$)** | **Max sOUR time (d)** | **Final COC ($g\ O_2\ kg^{-1}dm$)** | **Max conidia production (conidia $g^{-1}dm$)** | **Max prod time (d)** | **COC at max prod time ($g\ O_2\ kg^{-1}dm$)** |
|---|---|---|---|---|---|---|
| ***Preliminary test BB** | >0.78 | 1.92-4.7 | "4.76" | $7.0x10^8$ | 7.5-8 | 0.70-2.68 |
| **First TC BB** | 0.64 | 2.81 | 4.56 | $5.0x10^8$ | 7.5-8 | 3.01 |
| **Mixing BB** | 0.93 | 1.54 | 3.76 | $9.0x10^8$ | 7.5-8 | 3.76 |
| **DoE BB 1** | 1.55 | 1.11 | 6.40 | $1.9x10^9$ | 7.5-8 | 6.40 |
| **DoE BB 2** | 0.54 | 0.87 | 1.85 | $1.6x10^9$ | 7.5-8 | 1.85 |
| **Second TC BB** | 0.37 | 1.67 | 2.86 | $9.0x10^8$ | 7.5-8 | 2.08 |
| **Preliminary test TH** | 0.87 | 2.07 | 4.09 | $8.4x10^8$ | 7.5-8 | 1.07 |
| **First TC TH** | 0.64 | 3.75 | 4.59 | $1.9x10^9$ max<br>$1.3x10^9$ stable | 5.5-6 | 3.15 |
| **Mixing TH** | 0.94 | 2.09 | 3.24 | $9.0x10^8$ | 5.5-6 | 3.24 |
| **DoE TH 1** | 0.91 | 1.69 | 2.62 | $1.3x10^9$ | 5.5-6 | 2.62 |
| **DoE TH 2** | 0.46 | 0.92 | 1.66 | $2.0x10^9$ | 5.5-6 | 1.66 |
| **Second TC TH** | 0.44 | 2.6 | 2.71 | $1.4x10^9$ | 5.5-6 | 2.08 |

TC: time course, DoE: design of experiments; sOUR: specific oxygen uptake rate; COC: cumulative oxygen consumption; *sOUR data in preliminary test BB was lost between days 1.92 and 4.7 (see Figure 4.5 a), presented values are approximate. Results in mixing rows correspond to the best mixing reactor.

## Final remarks

Conidia production by BB and TH using rice husk in 0.5 L SSF PBBs has been optimized. 65-70% moisture, $5.5x10^6$ conidia $g^{-1}$dm inoculum concentration, 20 mL/min airflow, 25ºC temperature and 40 C/N ratio were BB optimum values. Same values were obtained with TH except for moisture (55-60%) and C/N ratio (25-55). Mixing was positive to TH conidia production when performed at 24h or 48h after inoculation. The robustness of the process shown through Box-plots allows establishing a highly probable conidia production range valid both for BB and TH ($5.0x10^8$-$1.3x10^9$ conidia $g^{-1}$dm). Results obtained in this chapter have been used as a base work for a reliable scale-up of the process, as well as serving as a starting point for the application of several operational techniques presented in following chapters.

# Chapter 5

## Definition of significant substrate parameters on fungal conidia production

This chapter has been published in: Sala, A., Vittone, S., Barrena, R., Sánchez, A., Artola, A., 2021. Scanning agro-industrial wastes as substrates for fungal biopesticide production: use of *Beauveria bassiana* and *Trichoderma harzianum* in solid-state fermentation. Journal of Environmental Management. 295. 113113.

## 5.1. Summary/Overview

As presented in Chapter 4; rice husk has proven as suitable substrate for both BB and TH conidia production. However, previous reported results in other works using tray or packed bed configuration achieved superior conidia production, albeit using different substrates such as rice, refused potatoes and sugarcane bagasse or straw and wheat bran (Xie et al., 2012; Santa et al., 2005; Zhang and Yang., 2015). These substrates present different characteristics that could make them more suitable for fungal conidia production when compared to rice husk. As such, residues presenting higher potential biodegradability than rice husk while being low enough to prevent further scale-up drawbacks. (Krishania et al., 2018) such as problems associated with heat transfer throughout the bed should be tested.

Most of the studies on fungal conidia production in Chapter 1 are focused on the optimization of a specific residue or on the selection of the best among a certain pool, without going in depth into a detailed analysis on the specific conditions which made a residue favourable when compared to others. A better understanding of the characteristics of a residue to be more suitable for fungal growth (both for BB and TH) is needed. It must also be considered that what might be suitable for one specific fungal strain might not be the best option for another, especially when they do not share growth and conidiation characteristics, as it has been observed in Chapter 4 for BB and TH.

As presented in Chapter 1; fungal SSF process parameters (temperature, moisture, pH, among others) have been optimized at laboratory scale by several authors using different statistical methods: Zhang and Yang (2015) used an orthogonal test to optimize TH conidia production; Bhanu Prakash et al. (2008) used response surface methodology to optimize three factors (pH, moisture and supplemented yeast extract) affecting *Metarhizium anisopliae* conidia production and Chapter 4 in this thesis has presented DoE to optimize five parameters (moisture, temperature, inoculum concentration, C/N ratio and airflow) affecting BB and TH conidia production. All these methods have focused on the optimization of certain parameters using a previously chosen substrate as base. However, none of them has analyzed data extracted from several substrates not with the aim of optimizing conidia production but to go deep into the reasons for a substrate being more suitable than other for a certain strain's conidia production. Due to the amount of data needed to accomplish this analysis, multivariate analysis tools such as principal component analysis (PCA) can be applied. PCA has been widely used for data reduction

in several areas such as chemometrics, food industry or clinical analysis (Kemsley et al., 2019., Wilson et al., 2019).

Fermentations in this Chapter were performed in collaboration with a student from Advanced Biotechnology UAB master degree (Silvana Vittone).

## 5.2. Materials

A total of 9 agro-industrial wastes being rice husk (RH); apple pomace (AP); whisky draff (WD); soy fiber (SF); rice fiber (RF); wheat straw (WS); beer draff (BD); orange peel (OP) and potato peel (PP) were used as substrates to test their feasibility for fungal BB and TH conidia production. Characterization of all raw materials is presented in Table 5.1 alongside mixture proportion charged in the reactors when needed. Rice husk was used as bulking agent in all mixtures. RH4 was used as rice husk supply for the screening tests.

**Table 5.1.** Raw material characterization of substrates studied in screening tests.

| Parameter/Substrate | RH4 | AP | WD | SF | RF |
|---|---|---|---|---|---|
| **MC (%)** | 9.9 ± 0.1 | 82.0 ± 2.4 | 75.0 ± 1.9 | 77.9 ± 1.7 | 66.7 ± 2.0 |
| **OM (%)** | 83.4 ± 1.7 | 89.3 ± 0.7 | 90.1 ± 1.8 | 96.2 ± 1.0 | 98.7 ± 0.5 |
| **pH** | 6.2 ± 0.2 | 4.79 ± 0.3 | 6.56 ± 0.3 | 7.40 ± 0.3 | 5.38 ± 0.2 |
| **CD (µs·cm$^{-1}$)** | 588 ± 40 | 1019 ± 62 | 146 ± 11 | 1179 ± 55 | 653 ± 32 |
| **Carbon (%)** | 40.4 ± 0.5 | 42.4 ± 0.7 | 49.7 ± 1.3 | 48.4 ± 0.2 | 48.9 ± 0.3 |
| **Hydrogen (%)** | 5.2 ± 0.2 | 6.6 ± 0.1 | 6.8 ± 0.2 | 6.9 ± 0.1 | 6.7 ± 0.1 |
| **Nitrogen (%)** | 0.4 ± 0.1 | 0.5 ± 0.1 | 4.4 ± 1.0 | 4.0 ± 0.1 | 10.1 ± 0.5 |
| **Sulphur (%)** | <0.1 | <0.1 | 0.2 ± 0.03 | 0.5 ± 0.01 | 0.5 ± 0.01 |
| **C/N ratio** | 95.3 ± 13 | 86.6 ± 4.1 | 11.7 ± 2.5 | 12.2 ± 0.4 | 4.8 ± 0.2 |
| **BD (kg m$^{-3}$)** | 166 ± 1 | 434 ± 3 | 378 ± 4 | 645 ± 9 | 561 ± 11 |
| **AFP$_R$ (%)** | 89.8 ± 1.2 | 58.3 ± 0.8 | 67.8 ± 1.5 | 45.1 ± 1.1 | 48.7 ± 1.0 |
| **TSC (mg g$^{-1}$dm)** | 17.9 ± 0.2 | 163.8 ± 7.1 | 105.2 ± 3.7 | 22.1 ± 0.6 | 22.1 ± 0.4 |

MC: moisture content; OM: organic matter; CD: conductivity; BD: bulk density; AFP$_R$: air-filled porosity; TSC: total sugar content; RH: rice husk; AP: apple pomace; WD: whisky draff; SF: soy fiber; RF: rice fiber.

**Table 5.1. (cont)** Raw material characterization of substrates studied in screening tests.

| Parameter/Substrate | WS | BDr | OP | PP |
|---|---|---|---|---|
| **MC (%)** | 8.23 ± 0.2 | 80.3 ± 3.1 | 78.8 ± 0.9 | 89.0 ± 0.2 |
| **OM (%)** | 93.5 ± 1.7 | 96.2 ± 1.3 | 96.8 ± 0.7 | 81.3 ± 1.1 |
| **pH** | 6.12 ± 0.4 | 6.14 ± 0.3 | 4.65 ± 0.3 | 6.97 ± 0.3 |
| **CD (µs $cm^{-1}$)** | 441 ± 25 | 175 ± 18 | 660 ± 20 | 314 ± 16 |
| **Carbon (%)** | 45.0 ± 0.1 | 48.9 ± 0.8 | 43.4 ± 1.3 | 43.0 ± 0.6 |
| **Hydrogen (%)** | 5.9 ± 0.1 | 7.0 ± 0.3 | 6.2 ± 0.1 | 5.8 ± 0.2 |
| **Nitrogen (%)** | 0.5 ± 0.1 | 4.8 ± 1.1 | 1.1 ± 0.1 | 1.8 ± 0.1 |
| **Sulphur (%)** | <0.1 | 0.1 ± 0.01 | <0.1 | <0.1 |
| **C/N ratio** | 85.4 ± 4.1 | 10.6 ± 2.5 | 41.1 ± 2.7 | 23.8 ± 0.9 |
| **BD (kg $m^{-3}$)** | 189 ± 2 | 368 ± 4 | 437 ± 8 | 450 ± 5 |
| **$AFP_R$ (%)** | 89.4 ± 1.7 | 66.0 ± 2.1 | 59.8 ± 0.7 | 59.5 ± 1.5 |
| **TSC (mg $g^{-1}$dm)** | 17.7 ± 0.3 | 120.0 ± 4.4 | 241.7 ± 9.5 | 40.8 ± 2.4 |

MC: moisture content; OM: organic matter; CD: conductivity; BD: bulk density; $AFP_R$: air-filled porosity; TSC: total sugar content; RH: rice husk; WS: wheat straw; BDr: beer draff; OP: orange peel; PP: potato peel.

## 5.3. Tests

### 5.3.1. Conidia production in triplicate batches

Triplicate SSF tests were performed with each substrate using both strains as inoculum. Tests were undertaken in SSF system 1 (0.5 L reactors). As stated in section 3.1.3, some substrates were mixed with rice husk to enhance their initial $AFP_R$. Table 5.2 summarizes initial values for main parameters of the different mixtures.

**Table 5.2.** Initial parameter values for substrates/mixtures used in scanning tests.

| Parameter / Substrate | MC (%) | OM (%) | pH | CD (µs $cm^{-1}$) | $AFP_R$ (%) | Mixture proportion (w:w) (%:%) (RH:other) |
|---|---|---|---|---|---|---|
| **RH** | 62.2 ± 0.4 | 83.5 ± 0.5 | 5.6 ± 0.2 | 465 ± 16 | 85.7 ± 2.2 | 100:0 |
| **AP** | 73.7 ± 0.5 | 89.3 ± 0.7 | 5.1 ± 0.1 | 917 ± 57 | 60.2 ± 1.8 | 20:80 |
| **WD** | 76.4 ± 1.1 | 96.9 ± 1.8 | 5.8 ± 0.1 | 513 ± 20 | 68.2 ± 1.1 | 5:95 |
| **SF** | 74.2 ± 2.5 | 92.2 ± 1.5 | 6.6 ± 0.2 | 1234 ± 18 | 45.4 ± 1.2 | 5:95 |
| **RF** | 66.3 ± 0.3 | 95.6 ± 1.0 | 5.6 ± 0.1 | 773 ± 49 | 48.8 ± 0.9 | 5:95 |
| **WS** | 67.0 ± 4.0 | 96.6 ± 1.3 | 5.8 ± 0.1 | 896 ± 4 | 84.4 ± 1.7 | 0:100 |
| **BDr** | 79.9 ± 3.4 | 95.0 ± 0.5 | 5.5 ± 0.3 | 266 ± 65 | 65.2 ± 1.3 | 5:95 |
| **OP** | 84.2 ± 0.8 | 94.6 ± 1.9 | 4.6 ± 0.4 | 660 ± 20 | 59.8 ± 1.2 | 0:100 |
| **PP** | 91.1 ± 0.7 | 81.6 ± 1.2 | 5.4 ± 0.3 | 419 ± 42 | 59.4 ± 0.7 | 0:100 |

MC: moisture content; OM: organic matter; CD: conductivity; $AFP_R$: air-filled porosity; RH: rice husk; AP: apple pomace; WD: whisky draff; SF: soy fiber; RF: rice fiber; WS: wheat straw; BDr: beer draff; OP: orange peel; PP: potato peel.

### 5.3.2. Principal component analysis (PCA)

PCA was performed as statistical approach to better understand the effect and relevance that several process parameters have in BB and TH fungal conidia production. PCA was performed using data of the following parameters collected in all fermentations: conidia production, initial moisture, initial pH, C/N ratio, total sugar content, $AFP_R$ and COC at 7 days (BB) or 5 days (TH), using Minitab 17 (Minitab Ltd) software. All values except for conidia production and COC corresponded to initial parameters of the used substrate/mixture. Analysis was performed for BB and TH data separately.

## 5.4. Results and discussion

### 5.4.1. Conidia production and respirometric analysis

Conidia production results for all substrates are shown in Fig. 5.1. Seven out of 10 substrates achieved productions of at least two orders of magnitude above inoculum concentration ($6.6x10^6$ conidia $g^{-1}$dm) with both strains. $1x10^9$ conidia $g^{-1}$dm was chosen as comparative value to test conidia production with all substrates. This value corresponds to half the maximum conidia production obtained in Chapter 4.

When working with BB, potato peel surpassed $1x10^9$ conidia $g^{-1}$dm, producing nearly $1.3x10^9$ conidia $g^{-1}$dm, being significantly different from the rest of productions with the only exception of rice husk. However, TH production nearly reached $1x10^9$ conidia $g^{-1}$dm with all substrates. Higher values were obtained with whisky draff ($3.2x10^9$ conidia $g^{-1}$dm), orange peel ($5.2x10^9$ conidia $g^{-1}$dm), potato peel ($6.4x10^9$ conidia $g^{-1}$dm) and beer draff ($7.5x10^9$ conidia $g^{-1}$dm). No significant differences in terms of conidia production were found between them. In the cases of soy and rice fiber, both substrates did not produce conidia above the inoculum concentration, neither for BB nor for TH. Obtained BB results are similar to those achieved in Chapter 4 using rice husk as substrate. However, TH conidia productions with most of the presented substrates are higher. In comparison, all substrates which showed conidia production achieved better results with TH than with BB, with the sole exception of rice husk. These results show TH as a more versatile fungus for conidia production, capable of achieving higher growth and sporulation in many different substrates when compared to BB. Similarly, Mishra et al. (2016) obtained $4.4x10^8$ conidia $g^{-1}$dm using rice husk and Santa et al. (2005) achieved $2.0x10^9$ conidia $g^{-1}$dm using refused potatoes (both of them with BB) and Mishra and Sundari (2017) obtained $2.0x10^8$ conidia $g^{-1}$dm with TH using wheat straw. Therefore, the rest of the presented substrates (apple pomace, whisky draff, soy fiber, rice fiber, beer draff and orange peel) had never been used for fungal conidia production using neither BB nor TH prior to this study. Also, potato peel (or similar potato residues) has not been found as substrate for TH conidia production. Other *Trichoderma* strains had been tested using rice husk as substrate in combination with polyurethane foam (Barrera et al., 2019), albeit in a different scale and type of reactor than the used in this chapter. Visual appearance of all substrates at the end of the fermentation are shown in Figure 5.2 (BB) and Figure 5.3 (TH).

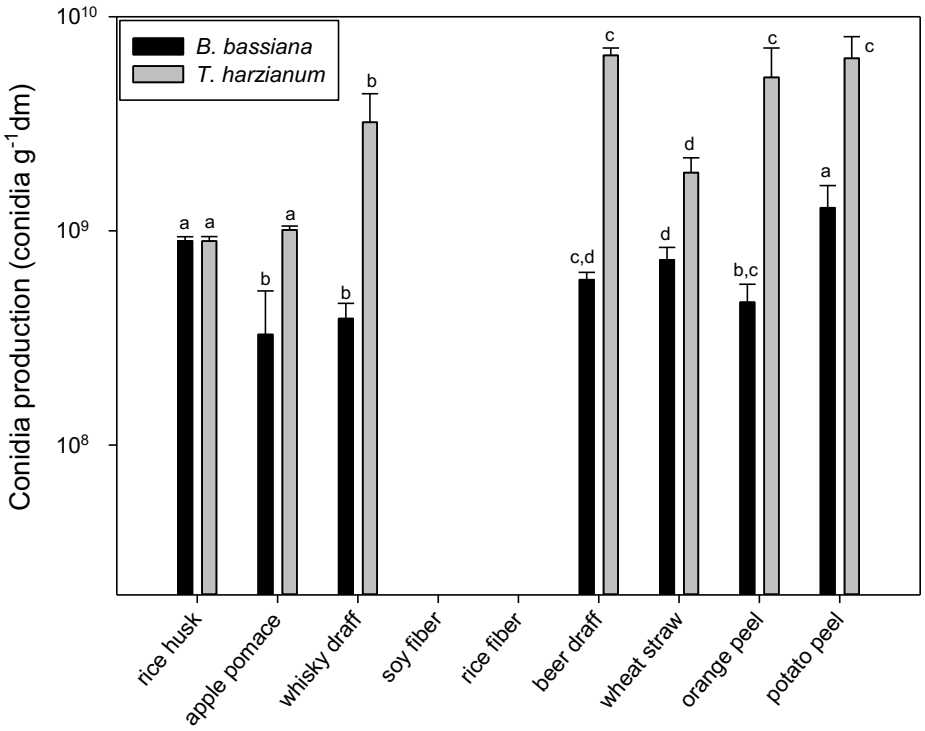



**Figure 5.1.** Conidia production obtained in substrate screening tests.


**Figure 5.2.** Appearance of all BB fermented substrates in substrate screening tests.


**Figure 5.3.** Appearance of all TH fermented substrates in substrate screening tests.

Figure 5.4 shows sOUR profiles for all substrates, Figure 5.4 a) for BB and Figure 5.4 b) for TH. sOUR profiles were different among all substrates, showing different potential biodegradability. BB sOUR profiles reached their maximum between days 1.5 and 3, while for TH it can be observed between days 1 and 2.5. These differences were also seen in the lag phase, which ends 12 h later in BB than in TH for most of the analysed substrates, as observed in Chapter 4. However, higher respiration indexes were not correlated to higher conidia production, as substrates like soy and rice fiber did not produce fungal conidia even though their sOUR maximum values were the highest with both strains. Having discarded inoculation and manipulation as possible contamination sources, we hypothesized that contamination came from the substrate. Nucleic acid analysis confirmed bacterial contamination in both fibres. Contamination identification is presented in detail in section 5.5.

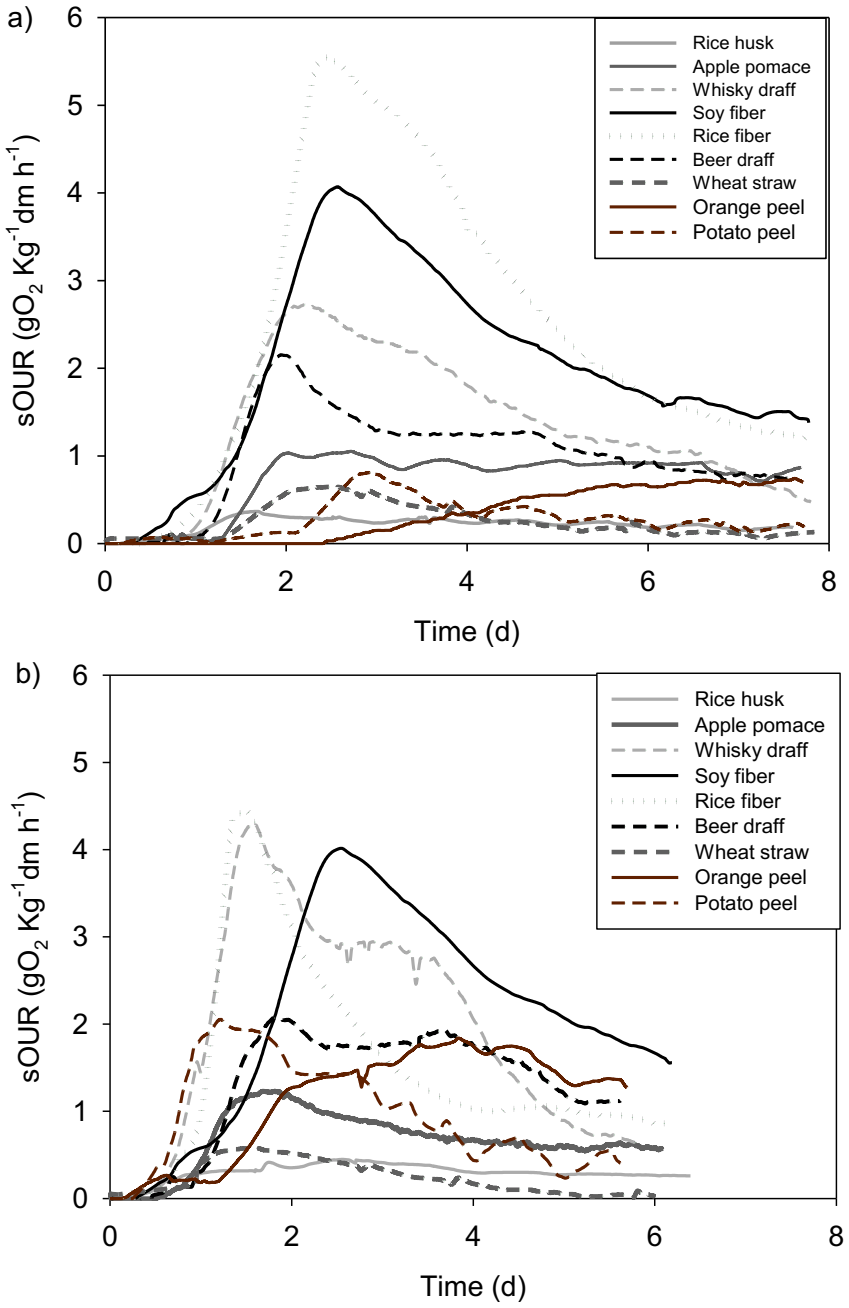



**Figure 5.4.** sOUR profiles obtained in substrate screening tests with all substrates. a) BB and b) TH.

Both potato peel (BB and TH) and orange peel (TH) did not present a particularly high respiration profile despite achieving high conidia production, while beer and whisky draff had higher respiration profiles coupled with also high conidia production when inoculating with TH. Some of the obtained respiration indexes, particularly those achieved with both fibres (soy and rice), are a bit off the panel in comparison to the rest. Values higher than 4 $gO_2$ $kg^{-1}$dm $h^{-1}$ were achieved. This might be due to the potential biodegradability of soy and rice fiber, which values were comparable to those achieved

using *Bacillus thuringiensis* (Ballardo, 2016) or the autochthonous microbial populations of the organic fraction of municipal solid waste (Marín, 2018) as inoculums. Nevertheless, and in accordance with Chapter 4 results, no correlation between conidia production and respiration indexes has been found, neither for BB nor for TH.

Table 5.3 (a and b) shows final parameters of substrate screening tests. Despite the use of rice husk to adjust $AFP_R$ (in the cases of apple pomace, whisky draff, soy fiber, rice fiber and beer draff), values for initial moisture were between 62 and 85% (91% exceptionally in the case of potato peel). Most of them were higher than the optimal for fungal growth found in Chapter 4 DoEs (60-70%). Final moisture values were slightly higher in most of the fermentations, which might be due to the use of closed batch reactors, where water produced during organic matter degradation accumulates. This behaviour in closed batch reactors might also have favoured the appearance of contamination. pH values were similar between strains and most of the substrates, with initial values between 5 and 6 and ending near 7–8, optimal values for fungal growth according to Papagianni (2004). Apple pomace and orange peel were the exception. While for apple pomace initial and final pH values were between 5.2 and 4.3 for both strains, values for orange peel were even lower, going from at 4.7 to 3.3 for BB but maintaining values higher than 4.0 in TH. These acidic values affected fungal growth and conidiation, as they maximize near neutral pH (Verma et al., 2007). In the case of BB, pH values around 6.0-7.0 have been reported as optimum for growth and conidiation, even finding acidic pH close to values of 3 as toxic (Santa et al., 2005; Padmavathi et al., 2003). Henceforth, acidic pH negatively affected BB, yielding the lowest conidia production obtained using BB with both substrates. As of TH, even though orange peel pH values reached 4.3 at the end of the fermentation, its conidia production was very high. This could mean that TH is able to tolerate such acidic pH, despite maximally growing near neutral pH (Verma et al., 2007). Consequently, the lowest conidia production with both strains was obtained using apple pomace and one of the lowest using orange peel with BB, despite both substrates having very high total sugar concentration when compared to the rest (Table 5.1).

**Table 5.3. (a)** Final parameter values obtained in substrate screening BB tests with all substrates.

| Parameter/ substrate | CP (conidia $g^{-1}dm$) | CPr (conidia $g^{-1}dm\ d^{-1}$) | CQ | MC (%) | pH | $COC_{7d}$ ($gO_2\ kg^{-1}dm$) |
|---|---|---|---|---|---|---|
| **RH** | $1.0x10^9$ | $1.3x10^8$ | 151 | 58.4 ± 1.8 | 6.9 ± 0.1 | 1.9 |
| **AP** | $3.3x10^8$ | $4.2x10^7$ | 50 | 73.9 ± 1.5 | 4.3 ± 0.8 | 4.7 |
| **WD** | $3.9x10^8$ | $5.0x10^7$ | 59 | 74.8 ± 0.2 | 7.7 ± 0.0 | 8.7 |
| **SF** | 0 | 0 | 0 | 78.6 ± 1.2 | 7.1 ± 0.1 | 14.7 |
| **RF** | 0 | 0 | 0 | 71.6 ± 0.9 | 7.5 ± 0.5 | 17.7 |
| **WS** | $7.3x10^8$ | $9.4x10^7$ | 111 | 69.4 ± 2.1 | 4.4 ± 0.2 | 1.6 |
| **BDr** | $5.9x10^8$ | $7.6x10^7$ | 89 | 79.6 ± 0.5 | 7.9 ± 0.1 | 15.1 |
| **OP** | $4.6x10^8$ | $5.9x10^7$ | 70 | 84.6 ± 0.5 | 3.3 ± 0.3 | 2.6 |
| **PP** | $1.3x10^9$ | $1.6x10^8$ | 197 | 91.4 ± 0.4 | 5.6 ± 0.2 | 2.6 |

CP: conidia production; CPr: conidia productivity; CQ: conidia quotient; MC: moisture content; $COC_{7d}$: cumulative oxygen consumption at 7 days; RH: rice husk; AP: apple pomace; WD: whisky draff; SF: soy fiber; RF: rice fiber WS: wheat straw; BDr: beer draff; OP: orange peel; PP: potato peel. Conidia production triplicate variation is shown in Figure 5.1.

**Table 5.3. (b)** Final parameter values obtained in substrate screening TH tests with all substrates.

| Parameter/ substrate | CP (conidia $g^{-1}dm$) | CPr (conidia $g^{-1}dm\ d^{-1}$) | CQ | MC (%) | pH | $COC_{5d}$ ($gO_2\ kg^{-1}dm$) |
|---|---|---|---|---|---|---|
| **RH** | $1.0x10^9$ | $1.7x10^8$ | 151 | 60.6 ± 1.6 | 7.5 ± 0.1 | 1.7 |
| **AP** | $1.0x10^9$ | $1.7x10^8$ | 151 | 74.6 ± 0.6 | 5.2 ± 0.2 | 3.8 |
| **WD** | $3.3x10^9$ | $5.7x10^8$ | 500 | 78.7 ± 0.7 | 8.1 ± 0.2 | 13.4 |
| **SF** | 0 | 0 | 0 | 79.3 ± 1.7 | 8.3 ± 0.1 | 9.5 |
| **RF** | 0 | 0 | 0 | 73.0 ± 1.7 | 8.4 ± 0.2 | 8.6 |
| **WS** | $1.9x10^9$ | $3.3x10^8$ | 288 | 70.9 ± 1.2 | 7.2 ± 0.1 | 1.5 |
| **BDr** | $7.5x10^9$ | $1.3x10^9$ | 1136 | 83.5 ± 0.5 | 7.4 ± 0.1 | 16.8 |
| **OP** | $5.2x10^9$ | $9.0x10^8$ | 788 | 83.3 ± 0.4 | 4.3 ± 0.4 | 6.8 |
| **PP** | $6.4x10^9$ | $1.1x10^9$ | 970 | 91.2 ± 0.3 | 7.0 ± 0.2 | 4.9 |

CP: conidia production; CPr: conidia productivity; CQ: conidia quotient; MC: moisture content; $COC_{5d}$: cumulative oxygen consumption at 7 days; RH: rice husk; AP: apple pomace; WD: whisky draff; SF: soy fiber; RF: rive fiber WS: wheat straw; BDr: beer draff; OP: orange peel; PP: potato peel. Conidia production triplicate variation is shown in Figure 5.1.

### 5.4.2. Principal component analysis (PCA)

Values for all analysed parameters in PCAs are presented in Table 5.4 (a and b). Both fibers results were not included in the analysis, as no BB nor TH conidia production was achieved using neither of them. This simplification allows avoiding possible noise in the analysis.

**Table 5.4. (a)** Values for all parameters used in BB PCA analysis.

| Parameter/ Substrate | CP (conidia $g^{-1}dm$) | MC (%) | pH | C/N ratio | TSC (mg $g^{-1}$ dm) | $AFP_R$ (%) | $COC_{7d}$ ($gO_2$ $kg^{-1}dm$) |
|---|---|---|---|---|---|---|---|
| **RH** | $1.0x10^9$ | 62.2 | 5.5 | 95.3 | 18.3 | 83.0 | 1.9 |
| **AP** | $3.3x10^8$ | 73.6 | 5.0 | 86.6 | 164.0 | 61.1 | 4.7 |
| **WD** | $3.9x10^8$ | 77.3 | 5.8 | 11.7 | 105.3 | 64.0 | 8.7 |
| **WS** | $7.3x10^8$ | 67.7 | 5.8 | 85.4 | 17.5 | 85.6 | 1.6 |
| **BDr** | $5.9x10^8$ | 78.6 | 5.7 | 23.5 | 120.4 | 64.3 | 15.1 |
| **OP** | $4.6x10^8$ | 83.2 | 4.7 | 41.1 | 241.7 | 59.7 | 2.6 |
| **PP** | $1.3x10^9$ | 91.1 | 5.5 | 23.8 | 40.8 | 61.4 | 2.6 |

CP: conidia production; MC: moisture content; TSC: total sugar content; $AFP_R$: air filled porosity; $COC_{7d}$: cumulative oxygen consumption at 7 days; RH: rice husk; AP: apple pomace; WD: whisky draff; WS: wheat straw; BDr: beer draff; OP: orange peel; PP: potato peel.

**Table 5.4. (b)** Values for all parameters used in TH PCA analysis.

| Parameter/ Substrate | CP (conidia $g^{-1}dm$) | MC (%) | pH | C/N ratio | TSC (mg $g^{-1}$ dm) | AFP (%) | $COC_{5d}$ ($gO_2$ $kg^{-1}dm$) |
|---|---|---|---|---|---|---|---|
| **RH** | $1.0x10^9$ | 62.3 | 5.7 | 95.3 | 18.3 | 83.0 | 1.7 |
| **AP** | $1.0x10^9$ | 73.8 | 5.1 | 86.6 | 164.0 | 61.1 | 3.8 |
| **WD** | $3.3x10^9$ | 75.4 | 5.9 | 11.7 | 105.3 | 64.0 | 13.4 |
| **WS** | $1.9x10^9$ | 66.2 | 7.2 | 85.4 | 17.5 | 85.6 | 1.5 |
| **BDr** | $7.5x10^9$ | 81.1 | 7.4 | 23.5 | 120.4 | 64.3 | 16.8 |
| **OP** | $5.2x10^9$ | 83.6 | 4.4 | 41.1 | 241.7 | 59.7 | 6.8 |
| **PP** | $6.4x10^9$ | 91.2 | 5.3 | 23.8 | 40.8 | 61.4 | 4.9 |

CP: conidia production; MC: moisture content; TSC: total sugar content; AFP: air filled porosity; $COC_{5d}$: cumulative oxygen consumption at 5 days; RH: rice husk; AP: apple pomace; WD: whisky draff; WS: wheat straw; BDr: beer draff; OP: orange peel; PP: potato peel.

As presented in Table 5.5 (a and b), the majority of the data variance is explained with 3 components, being superior to 90% for both BB and TH. When looking at the 2 principal components with higher % variance, at least 75% of the data variance is explained for BB and 83% for TH. With 2 principal components, a minimum data variance of 75% is explained in each analysis. The two principal components (PC1 and PC2) which presented higher variance for BB or TH were plotted against each other and presented in Figure 5.5 in biplot form: Fig. 5.5 a) shows BB results and Figure 5.5 b) for TH. The biplots are different between strains, implying that different parameters have relative relevance in conidia production depending on the chosen strain

**Table 5.5. (a)** Eigenvalues and variance obtained in BB PCA analysis. The 3 components presenting highest obtained eigenvalues are shown.

| PCA component | Eigenvalue | Variance (%) | Cumulative variance (%) |
|---|---|---|---|
| **PC1** | 3.317 | 0.474 | 0.474 |
| **PC2** | 1.917 | 0.274 | 0.748 |
| **PC3** | 1.370 | 0.196 | 0.943 |

PCA: principal component analysis; PC: principal component.

**Table 5.5. (b)** Eigenvalues and variance obtained in TH PCA analysis. The 3 components presenting highest obtained eigenvalues are shown.

| PCA component | Eigenvalue | Variance (%) | Cumulative variance (%) |
|---|---|---|---|
| **PC1** | 4.044 | 0.578 | 0.578 |
| **PC2** | 1.779 | 0.254 | 0.832 |
| **PC3** | 0.704 | 0.101 | 0.932 |

PCA: principal component analysis; PC: principal component.

When focusing on parameters which have a relevant effect on conidia production (plotted at less than 90º from conidia production), two different tendencies are clearly distinguished. On the one hand, BB is influenced by initial pH and $AFP_R$. On the other hand, TH is influenced by COC, initial moisture and total sugar content. Relevant differences between strains are highlighted, as no parameter is repeated. In the BB case, all relevant parameters are linked to a proper adaptation to the substrate to ensure correct fungal growth. This adaptation is more critical in BB compared to TH given its variable lag phase, which can last up to 2.5 days depending on the substrate as seen in Figure 5.2. Substrates which achieved conidia productions of at least $1x10^9$ conidia $g^{-1}$dm in BB (rice

husk and potato peel) were the ones which combined $AFP_R$ of at least 58% with initial pH close to 5.5 and C/N ratio near or superior to 25, even though the influence of C/N ratio in BB conidia production is not as clear as the rest. In comparison, all TH parameters are more related to the potential biodegradability of the substrate, achieving higher conidia production if the three parameters (COC, initial moisture and total sugar content) are high enough, as it happens with all substrates which yielded higher TH conidia production. Substrates which performed better in terms of conidia production all had initial moisture levels greater than 75% (even reaching values of 91%), COC of at least 5 $gO_2\ kg^{-1}dm$ (even reaching values of 16) and total sugar content of at least 40 mg $g^{-1}dm$ (and even of 242), whereas the rest of the tested substrates did not accomplish at least one of these limits.

All PCA analysis show similar substrate groups, even though they are located on different quadrants depending on the analysis. In all analysis, wastes are grouped independently of the most relevant parameters for sporulation. The following groups are observed: rice husks and wheat straws, all draff and potato peels, apple pomaces and orange pomaces.

Regarding the organization of the groups throughout the axis and the parameters which are presented in the components, they also vary between strains. In BB, PC1 presents a distribution by $AFP_R$ and sugars (biodegradability), showing that lower biodegradability is better for BB. However, in TH the groups are organized depending on their $AFP_R$ and conidia production, with the best residues in terms of conidia production being the two draff, orange peel and potato peel, corresponding to the ones with at least both high C/N ratio and high biodegradability (COC), as it is shown in PC2. It seems that while high biodegradability is an essential parameter for a substrate to yield the highest TH conidia production, it is clearly the opposite for BB, as substrates which present lower biodegradability are the ones which have yielded highest conidia production with this strain. These organizations also confirm the relevance of parameters linked to proper fungal growth and adaptation to the substrate on BB conidia production and also the relevance of parameters related to biodegradability of the substrate on TH conidia production. This behaviour is exemplified by moisture and $AFP_R$, always presented within 180◦, showing inverted relevance between strains.

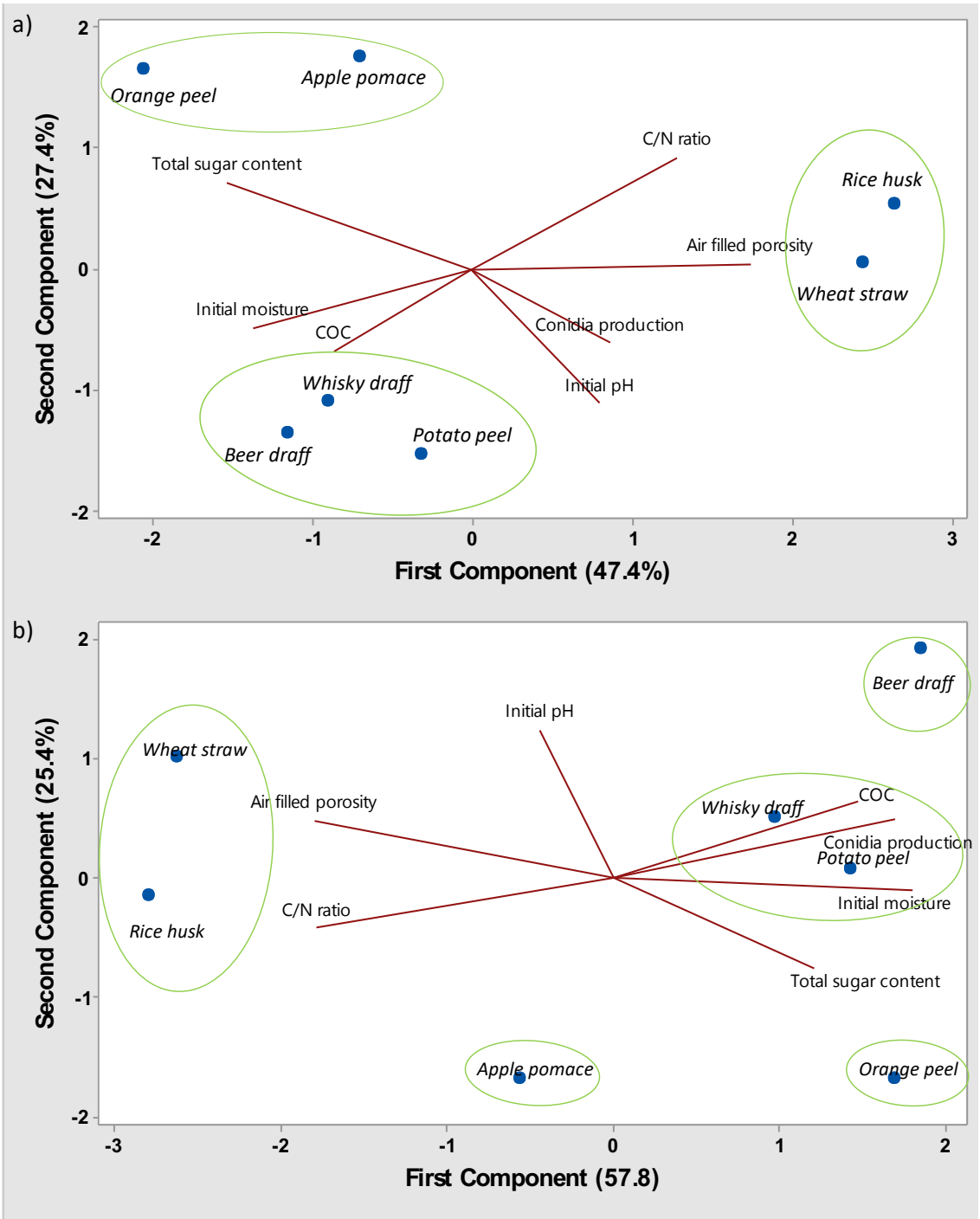



**Figure 5.5.** PCA plots (component 1 vs component 2) obtained in the PCA of all substrates (rice husk, apple pomace, whisky draff, wheat straw, beer draff, orange peel and potato peel) suitable for conidia production. a) BB and b) TH. Different substrate groups are shown with circles.

## 5.5. Contaminant identification

As shown both with respiration profiles (Figures 5.4 a and b) and final fermentation photos (Figures 5.2 and 5.3) observed growth using soy fiber and rice fiber did not correspond to BB or TH. Figure 5.6 shows results obtained in TH plate growth corresponding to productions for whisky draff (B) and rice fiber (C) compared to a pure inoculum culture (A). Comparison at microspore level with 100x augments between the same samples is also shown. While results obtained with whisky draff (B) can be compared to a certain extent with the inoculum (A) both at microscope and plate level, none of them are similar to the photographs corresponding to rice fiber (C). In this sample, it was not possible to visualize conidia using the 100x microscope augment. In addition, plate results did not seem to correspond to fungal growth but to a bacterial culture (presence of rod-shaped colonies showing no hyphae nor conidia).

Nucleic acid analysis confirmed that fermentations performed with both fibres were contaminated by the bacteria *Burkholderia gladioli* (BG). This genus is usually pathogenic for humans, plants and animals, although they usually live in symbiosis with plants or fungi, and can be found in plants such as rice, animals and soil (Stoyanova et al., 2007, Nandakumar et al., 2008). BG growth characteristics are similar to BB's and TH's, especially due to similar optimal temperatures but with faster BG growth (3-4 days for BG vs 6-8 days respectively for TH or BB) (Ross et al., 2014), favouring BG's growth over fungal conidia production. Even though all substrates were autoclaved prior to the fermentation, BG was still present in both fibres, probably taking advantage of the fungal inoculum to its own profit. This hypothesis is supported by the high respiration profiles shown both with BB and TH (Figure 5.2 a and b). Profiles which, as suspected, did not correspond to fungal culture respiration profile but to a bacterial culture. Low $AFP_R$ of the used substrates could favoured these results, leading to substrate compaction and highly difficulting fungal colonisation while favouring bacterial growth (Krishna, 2005; Krishania; 2018).

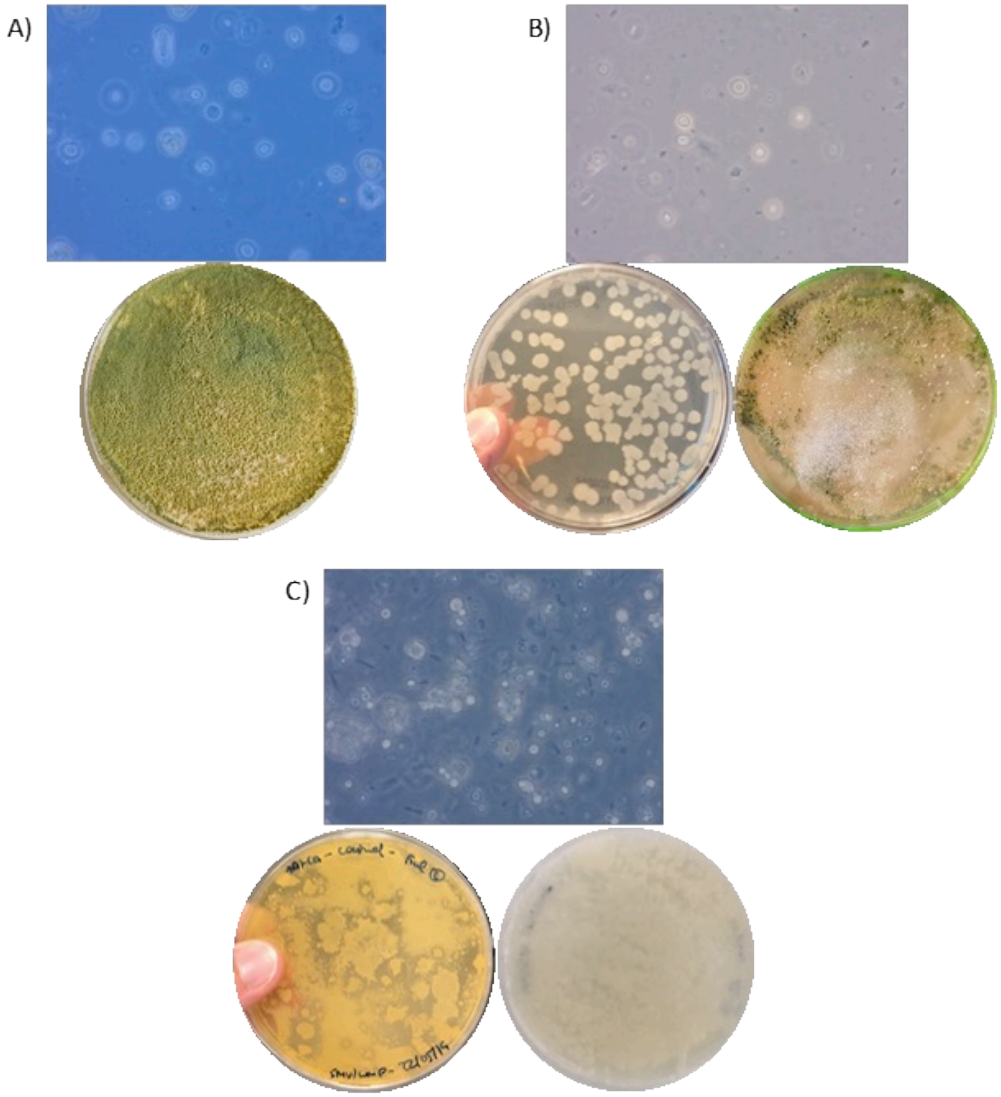



**Figure 5.6.** Microscope and plate growth images obtained with different TH cultures. A) pure inoculum cultures, B) whisky draff cultures and C) rice fiber cultures. In B) and C), 2 plates are shown: first plate corresponds to raw substrate extraction (before autoclaving) and second plate to fermented substrate extraction.

## 5.6. Global analysis

A brief comparison between strains in terms of conidia production and respirometric parameters is presented in this section.

In terms of conidia production and according to Figure 5.1; all substrates which showed conidia production above inoculum concentration achieved much higher conidia production (in most of the cases being of one order of magnitude above) with TH than with BB, with the sole exception of rice husk. These results position the used TH strain as a more versatile fungus for conidia production, capable of achieving higher growth and

conidiation in many different substrates when compared to the used BB strain. Such versatility could be expected, as the genera *Trichoderma* has been used as biological control of other fungi, weeds and bacteria, whereas the genera *Beauveria* pest control capabilities are heavily oriented to invertebrate pests (Verma et al., 2007; Mascarin and Jaronski, 2016).

In terms of respiration indexes and as presented in Figure 5.4; higher respiration indexes did not correspond to higher conidia production in neither of the tested strains. These results are consequent with presented results on conidia production and respiration indexes in Chapter 4 (section 4.5) using rice husk as substrate with the same fermentation system. When working with BB and TH, respiration indexes show no correlation with conidia production, regardless of the chosen substrate. However, respiration profiles in Figure 5.2 and final fermentation photos (annex) demonstrate correlation between respiration indexes and fungal growth, as substrates which exhibited higher hyphae growth where the ones achieving higher respiration indexes. This behaviour is also independent of the used strain. Correlation between fungal growth and respiration indexes using BB or TH has been previously presented (Santa el al., 2005; Lopez-Ramirez et al., 2018).

## Final remarks

Rice husk, apple pomace, whisky draff, beer draff, wheat straw, orange peel and potato peel could be successfully used as substrates for fungal conidia production. Rice husk and potato peel are more suitable for *Beauveria bassiana* (BB) whereas beer draft, orange peel and potato peel are more suitable for *Trichoderma harzianum* (TH). Soy fiber and rice fiber were discarded due to bacterial contamination, which was resistant to autoclave and further enhanced by low $AFP_R$ values. According to PCA, relevant BB parameters (initial pH and $AFP_R$) link to proper adaptation to the substrate to ensure fungal growth, while relevant TH parameters (COC, initial moisture and total sugar content) relate to potential substrate biodegradability. Thus, valorisation of the studied residues trough SSF has been demonstrated feasible, also highlighting the main waste properties and process parameters for the two fungi used. These results can be the starting point to explore this process as a recovery option for other agro-industrial residues. Additionally, other BB and TH isolates could be tested for further validation of the presented methodology. Finally, these results serve as a basis to choose substrate

alternatives to rice husk to perform and scale-up SSF conidia production, which will be presented in depth in further chapters in this thesis.

# Chapter 6

## PBB production strategies and scale-up to 22L

Part of this chapter has been published in: Sala, A., Barrena, R., Sánchez, A., Artola, A., 2021. Fungal biopesticide production: process scale-up and sequential batch mode operation with *Trichoderma harzianum* using agro-industrial solid wastes of different biodegradability. Chemical Engineering Journal. 425. 131620.

## 6.1. Summary/Overview

In this chapter, fungal SSF process scale-up in PBB and enhancement via the application of sequential-batch reactor (SBR) operational strategies are addressed, both with BB and TH as inoculum.

As already stated, the most used reactor design for fungal SSF conidia production is the tray bioreactor. This design has achieved the best results when scaling-up (Thomas et al. 2013), making it the preferred design at commercial scale (Krishania et al., 2018; Mascarin et al., 2019). However, PBBs development has gained relevance in the recent years due to easier operational handling in comparison to tray bioreactors (Krishania et al., 2018). In the last years, some authors have reported various SSF fungal conidia production processes using PBBs at different scales (Santa et al., 2005; Barrera et al., 2018; Lopes-Perez et al., 2019; Méndez-González et al., 2020), however, research on the topic is still scarce.

Interesting strategies such as gas-double dynamic fermentation (Liu et al., 2018) or the use of inner supports to enhance fungal growth (Barrera et al., 2019) have been developed to enhance conidia production when working with tray bioreactors. However, PBBs conidia production related articles are still improving the traditional batch operation. One promising path to improve PBBs conidia production lies on SBR operation. This strategy eliminates the requirements of fresh inoculum for each batch (reducing the quantity of required inoculum to process the same amount of substrate). It has been successfully applied in the GICOM group to produce cellulases (Cerda, 2017) in a pilot scale reactor of 50 L or to produce aroma compounds using sugarcane bagasse in a 22 L reactor (Martínez, 2018).

SSF scale-up difficulties must also be taken into consideration when working with PBBs. As presented in section 1.4, numerous problems must be faced when scaling up SSF processes, particularly those related to heat generation and mass transfer thorough the bed (Pandey, 2003; Cerda, 2017; Soccol et al., 2017; Mejías, 2020). These problems are more difficult to overcome in comparison to working with tray bioreactors due to superior bed thickness (Krishania et al., 2018). This issue must be addressed from the very beginning of the process at substrate selection level, as generated heat varies significantly depending on substrate biodegradability. Substrates presenting naturally high $AFP_R$ such as rice husk can be used without mixing with a bulking agent.

Out of all tested residues in Chapter 5, beer draff conidia production was promising, specially using TH. When using beer draff, correct $AFP_R$ adjustment with the addition of a bulking agent is mandatory. In addition, sufficient flow of saturated air must be supplied, as it also helps at removing part of the generated metabolic heat (Pandey et al., 2008). Apart from $AFP_R$ differences, beer draff also presents much higher total sugar content than rice husk, meaning more carbon and nitrogen is available for fungal growth and conidiation. Moreover, it is an easy to obtain agro-industrial waste as it is produced as a waste of the brewery industry, one of the most productive industries of beverages in the world (Aliyu and Bala, 2011). This industry generates large quantities of brewer's spent grain or beer draff, a solid leftover obtained after the fermentation process, which is primarily used as animal feed (Ibarruri et al., 2019). At the moment of this thesis writing and to the author's knowledge, beer draff has not been previously used as conidia substrate neither for BB nor for TH conidia production. However, it has been used as substrate to produce enzymes and polyhydroxyalkanoates using AN (Llimós et al., 2020), implying novelty in its use as substrate.

The aims of this Chapter are: i) to develop a robust, reproducible and scalable process for BB and TH conidia production in packed bed reactors using agro-industrial wastes as substrates, ii) to present and adapt SBR as a feasible operation strategy to substitute traditional batch in packed bed TH fungal growth and iii) to test SBR using substrates presenting different biodegradability and $AFP_R$ in order to compare its effect towards the reactors' temperature. As a clarification, when referring to scale-up in this work we are always referring to bench scale (up to 22L), as no tests were performed at volumes which could be considered preindustrial or demonstration scales.

## 6.2. Materials

A total of two different rice husk supplies and two different beer draff supplies were used to perform all tests in this chapter, its characterization is summarized in Table 6.1. RH4 was the same supply presented in Chapter 4 and BDr1 was the same supply used in Chapter 5. Bulking agent (wood chips) characterization is also shown. Substrate supplies used in each test are indicated in the correspondent 6.3 section.

**Table 6.1.** Characterization of substrates and bulking agent used in production strategies and scale-up tests.

| Parameter/ supply | RH4 | RH5 | RH Mean values | BDr1 | BDr2 | BDr Mean values | WC1 |
|---|---|---|---|---|---|---|---|
| **MC (%)** | 9.9±0.1 | 10.9±0.1 | 10.3 | 80.3±3.1 | 72.5±2.3 | 76.4 | 9.7±0.3 |
| **OM (%)** | 78.2±1.7 | 86.2±3.0 | 82.6 | 94.3±0.8 | 92.8±0.2 | 93.6 | 98.4±0.6 |
| **pH** | 6.2±0.2 | 5.9±0.3 | 5.9 | 6.1±0.3 | 6.9±0.2 | 6.5 | 4.6±0.2 |
| **Carbon (%)** | 40.4±0.5 | 40.6±1.0 | 40.3 | 48.9±0.8 | 47.5±0.4 | 48.2 | 46.9±0.6 |
| **Hydrogen (%)** | 5.2±0.2 | 5.3±0.2 | 5.2 | 7.0±0.3 | 6.8±0.1 | 6.9 | 6.2±0.3 |
| **Nitrogen (%)** | 0.4±0.1 | 0.5±0.03 | 0.4 | 4.8±1.1 | 3.27±0.2 | 4.04 | 0.4±0.2 |
| **Sulphur (%)** | <0.1 | <0.1 | <0.1 | 0.1±0.01 | 0.1±0.02 | 0.1 | <0.1 |
| **C/N ratio** | 95.3±13 | 75.4±3.4 | 85.4 | 10.6±2.5 | 14.6±0.7 | 12.6 | 117.4±13.6 |
| **BD (kg $m^{-3}$)** | 166±1 | 172±2 | 165.3 | 368±4 | 351±3 | 360 | 107±3 |
| **TSC (mg $g^{-1}$dm)** | 17.9±0.2 | 17.4±0.2 | 17.7 | 120.0±4.4 | 126.8±3.7 | 123.4 | 96.7±10.3 |
| **$AFP_R$ (%)** | 89.8±1.2 | 90.6±0.8 | 90.2 | 66.0±2.1 | 62.0±2.4 | 64.0 | 95.3±0.5 |

MC: moisture content; OM: organic matter; BD: bulk density; TSC: total sugar content; $AFP_R$: air-filled porosity; RH: rice husk; BDr: beer draff; WC: wood chips.

## 6.3. Tests

### 6.3.1. Experiments at 1.5 L scale

#### 6.3.1.1. Preliminary batches

Before the start of the SBR strategy test, preliminary batches were performed to test the effects of the scale-up on conidia production, productivity, and the rest of the parameters. Using RH4 or BDr1 as substrate, one triplicate was performed with each substrate and strain. When working with beer draff, $AFP_R$ and initial moisture were adjusted with the addition of wood chips. Fermentation time was variable between tests but always superior to the optimum conidia production time found in Chapter 4, due to

the possibility of optimal conidia production time variations when scaling. Initial parameters for these fermentations are presented in Table 6.2. If tested in Chapter 4, parameter values were kept as near as possible to the optimal values. The scale-up criterion (superficial velocity) ($m^3$ $s^{-1}$ $g^{-1}$dm), which has determined airflow value in the first triplicate.

**Table 6.2.** Initial parameter values for 1.5 L preliminary batches.

| Parameter/ batch | MC (%) | OM (%) | IC (conidia $g^{-1}$dm) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}$dm) | Mixture (BDr/WC) (w/w) | $AFP_R$ (%) |
|---|---|---|---|---|---|---|---|---|
| **RH** | 65.8± 2.3 | 84.7± 2.1 | $2.5x10^6$ | 5.5±0.1 | 60 | 0.40-0.58 | (-) | 82 |
| **BDr** | 65.2± 2.4 | 98.7± 0.5 | $7.3x10^6$ | 5.4±0.3 | 100 | 0.80–0.96 | 70/30 | 71 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; sAF: specific airflow; RH: rice husk; BDr: beer draff; WC: wood chips; $AFP_R$: air-filled porosity.

### 6.3.1.2. Sequential-batch reactor strategy 1

To test the feasibility of using SBR as a strategy to produce fungal conidia, a "traditional" SBR strategy (from now on strategy 1) was proposed in the model shown on Figure 6.1. Using RH5 as substrate, triplicate propagation reactors were grown and harvested at maximum conidiation time. Three new batches were loaded using extracted material of the propagation reactors as inoculum in 3 different proportions: 95% raw material +5% inoculum, 90% raw material +10% inoculum and 80% raw material +20% inoculum. To distribute inoculum as uniformly as possible through the raw material, inoculum was thoroughly mixed with it prior to the start of the second batches. Second set of loaded reactors was also harvested at maximum conidiation time. Analysis was performed with initial and final samples of each set. In addition, to evaluate airflow needs in the 1.5 L reactors, lower airflow rate was tested in comparison to preliminary tests. Initial parameter values of SBR strategy 1 fermentations are presented in Table 6.3.

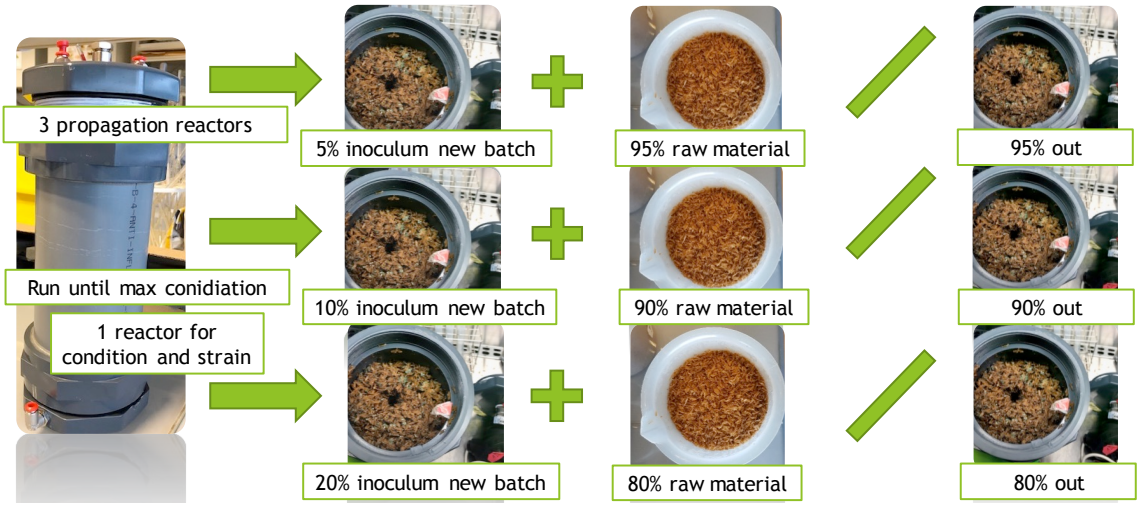



**Figure 6.1.** Schematic representation of SBR strategy 1 approach.

**Table 6.3.** Initial parameter values for 1.5 L rice husk SBR strategy 1 tests.

| Parameter | MC (%) | OM (%) | IC (conidia $g^{-1}$dm) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}$dm) |
|---|---|---|---|---|---|---|
| **Propag.** | 61.8 ± 5.0 | 84.1 ± 0.9 | $6.6x10^6$ | 5.7 ± 0.1 | 35 | 0.21 - 0.31 |
| **SBR** | 55.3 ± 1.2 | 84.5 ± 0.9 | Variable* | 5.9 ± 0.1 | 35 | 0.18 – 0.26 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; sAF: specific airflow; Propag: propagation reactors; SBR: sequential batch reactors.

### 6.3.1.3. Sequential-batch reactor strategy 2

SBR strategy 2 was proposed as an alternative to strategy 1 to test the feasibility of using liquid suspension extracted from the propagation reactor as inoculum for the next batch. Schematic representation of the model is shown in Figure 6.2. Triplicates were performed in all batches. RH5 or BDr 1 were used as substrate. Except for the first batch inoculum (obtained from plates as usual), inoculum for the following batches of the sequence was obtained by liquid extraction of fungal conidia contained in the solid material of the previous batch in the day determined through to inoculant age test (section 6.3.1.4). Conidia were extracted and diluted to $6.6x10^6$ conidia $g^{-1}$dm. Analysis were performed with initial and final samples of each set. Initial parameter values of SBR strategy 2 fermentations are presented in Tables 6.4 (rice husk) and 6.5 (beer draff).

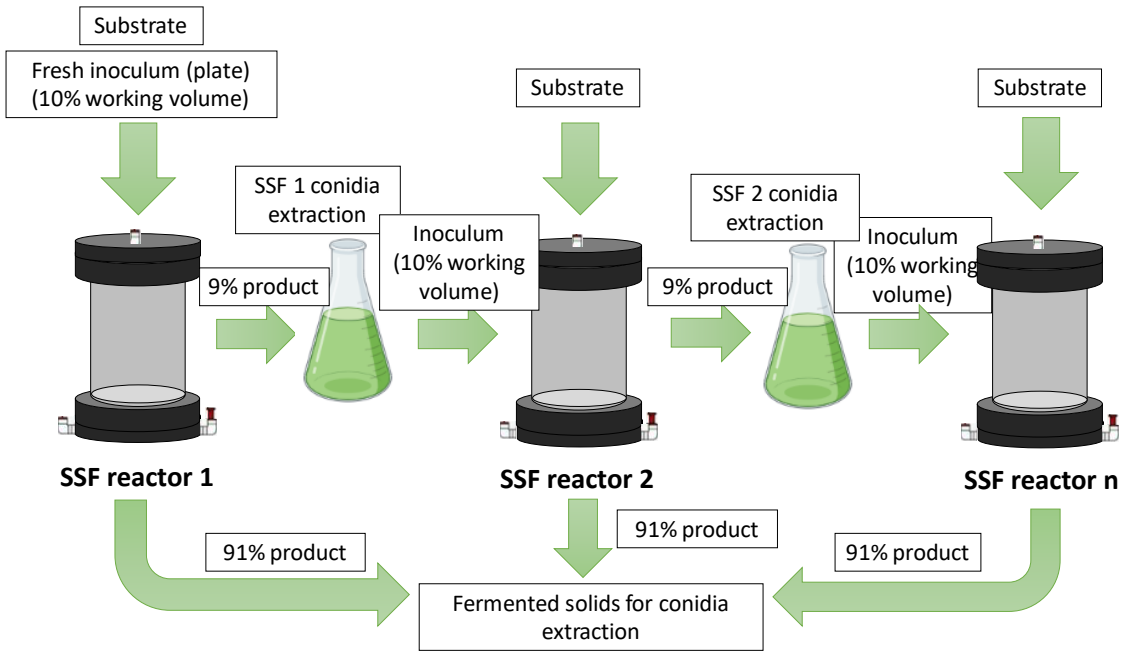



**Figure 6.2.** Schematic representation of SBR strategy 2 tests on 1.5L or 22L reactors. Some icons were provided by BioRender (https://biorender.com/).

**Table 6.4.** Initial parameter values for 1.5 L rice husk SBR strategy 2 tests.

| Parameter | MC (%) | OM (%) | IC (conidia $g^{-1}dm$) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}dm$) |
|---|---|---|---|---|---|---|
| **Batch 1** | 64.2±1.0 | 84.1±0.3 | $7.5x10^6$ | 6.1±0.3 | 35 | 0.22-0.33 |
| **Batch 2** | 56.6±1.1 | 85.4±0.6 | $6.2x10^6$ | 6.3±0.2 | 35 | 0.19–0.27 |
| **Batch 3** | 54.9±0.5 | 82.1±0.4 | $6.0x10^6$ | 6.6±0.2 | 35 | 0.18–0.26 |
| **Batch 4** | 54.8±0.6 | 81.6±0.5 | $6.0x10^6$ | 6.6±0.1 | 35 | 0.18–0.26 |
| **Batch 5** | 57.7±0.2 | 84.7±0.4 | $4.8x10^6$ | 6.6±0.2 | 35 | 0.19–0.28 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; sAF: specific airflow.

**Table 6.5.** Initial parameter values for 1.5 L beer draff SBR strategy 2 tests.

| Parameter | MC (%) | OM (%) | IC (conidia $g^{-1}dm$) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}dm$) |
|---|---|---|---|---|---|---|
| **Batch 1** | 65.2±2.4 | 98.7±0.5 | $7.3x10^6$ | 5.4±0.3 | 100 | 0.80-0.96 |
| **Batch 2** | 64.7±4.6 | 97.7±0.6 | $4.8x10^6$ | 5.7±0.2 | 100 | 0.79–0.95 |
| **Batch 3** | 60.7±1.3 | 98.1±0.3 | $3.5x10^6$ | 5.5±0.3 | 100 | 0.71–0.85 |
| **Batch 4** | 61.5±2.4 | 98.4±0.5 | $4.5x10^6$ | 5.7±0.3 | 100 | 0.72–0.87 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; sAF: specific airflow.

### 6.3.1.4. Inoculant age test

A test to determine the optimum day to extract conidia as inoculum for consequent SBR fermentations was performed using RH4 as substrate. Same test was proposed for BB and TH. Schematic representation of the test is presented in Figure 6.3. A 22 L rice husk batch was run for 9 days. Sampling of this reactor was performed in days 4, 6, 8 and 9. Sampling times were chosen according to results on optimal conidia production time obtained in Chapter 4. In all these samples (25 g each), conidia were extracted and used as inoculum for triplicate 0.5 L reactors using rice husk as substrate. To determine best inoculant age, conidia were counted at 8 (BB) or 6 (TH) days from the start of the 0.5 L reactors' fungal growth.

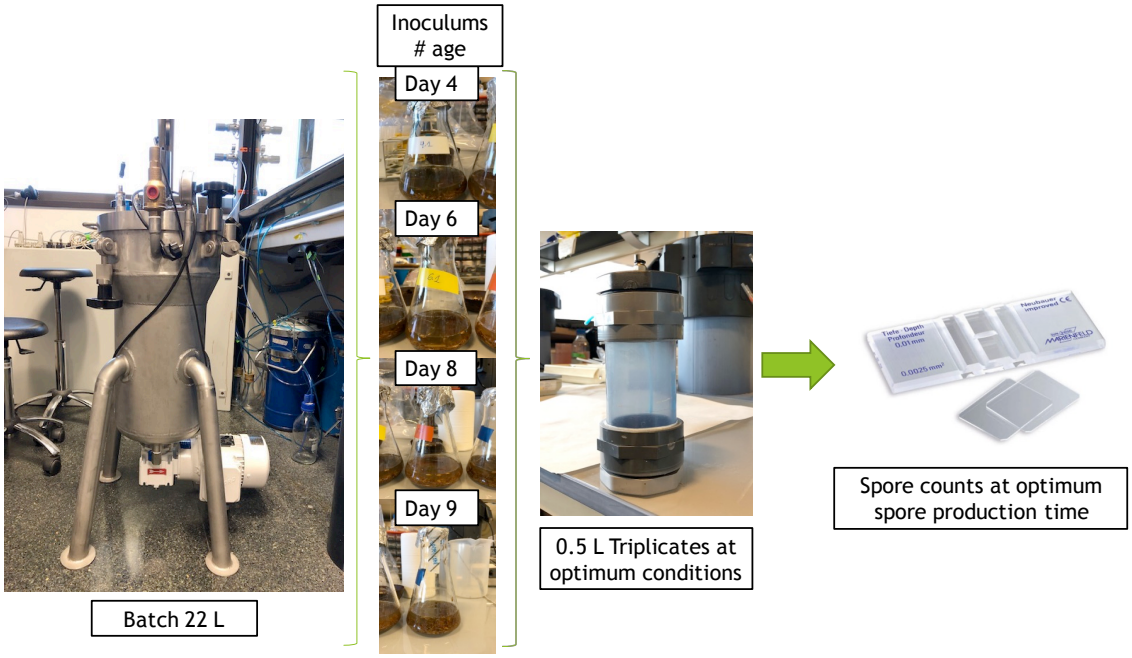



**Figure 6.3.** Schematic representation of inoculant age tests planning.

## 6.3.2. Experiments at 22 L scale

RH5 and BDr2 were used as substrates in all 22 L scale fermentations.

### 6.3.2.1. Preliminary batches

Prior to SBR strategy tests, preliminary batches were performed to test the scale-up effects on the process. Two different beer draff mixtures were tested. Initial parameter values, including beer draff mixture proportions, are presented in Table 6.6.

**Table 6.6.** Initial parameter values for 22 L preliminary batches.

| Parameter/ batch | MC (%) | OM (%) | IC (conidia $g^{-1}dm$) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}dm$) | Mixture (BDr/WC) (w/w) | $AFP_R$ (%) |
|---|---|---|---|---|---|---|---|---|
| **RH** | 63.2± 1.7 | 86.0± 2.3 | $6.8x10^6$ | 6.3± 0.3 | 500 | 0.31-0.45 | (-) | 82.5± 0.4 |
| **BDr (1)** | 65.8± 3.2 | 98.1± 0.3 | $5.8x10^6$ | 5.7± 0.3 | 1500 | 0.76-1.10 | 70/30 | 70.3± 0.9 |
| **BDr (2)** | 52.3± 1.6 | 97.4± 0.1 | $5.9x10^6$ | *6.5± 0.2 | 1500 | 0.54–0.79 | 40/60 | 81.2± 0.5 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; sAF: specific airflow; RH: rice husk; BDr: beer draff; WC: wood chips; $AFP_R$: air-filled porosity; *: modified value in 2nd BB preliminary batch.

### 6.3.2.2. Sequential-batch reactor

Same approach and analyses as presented for 1.5 L SBR fermentations were followed for 22 L SBR scale-up. Tables 6.7 and 6.8 present initial process parameters (rice husk and beer draff respectively).

**Table 6.7.** Initial parameter values for 22 L rice husk SBR strategy tests.

| Parameter | MC (%) | OM (%) | IC (conidia $g^{-1}dm$) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}dm$) |
|---|---|---|---|---|---|---|
| **Batch 1** | 58.7 ± 0.1 | 83.3 ± 0.7 | $6.4x10^6$ | 6.6 ± 0.2 | 500 | 0.28 – 0.40 |
| **Batch 2** | 60.4 ± 0.4 | 87.3 ± 0.5 | $6.6x10^6$ | 6.9 ± 0.1 | 500 | 0.29 – 0.42 |
| **Batch 3** | 57.3 ± 0.2 | 87.5 ± 0.8 | $6.2x10^6$ | 7.0 ± 0.2 | 500 | 0.27 – 0.39 |
| **Batch 4** | 59.5 ± 1.2 | 85.9 ± 0.4 | $6.5x10^6$ | 6.7 ± 0.2 | 500 | 0.28 – 0.41 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; sAF: specific airflow.

**Table 6.8.** Initial parameter values for 22 L beer draff SBR strategy tests.

| Parameter | MC (%) | OM (%) | IC (conidia $g^{-1}dm$) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}dm$) |
|---|---|---|---|---|---|---|
| **Batch 1** | 51.1 ± 1.3 | 98.7 ± 0.7 | $6.1x10^6$ | 4.4 ± 0.2 | 1500 | 0.53 - 0.77 |
| **Batch 2** | 55.9 ± 3.1 | 97.5 ± 0.4 | $6.1x10^6$ | 4.8 ± 0.3 | 1500 | 0.59 – 0.85 |
| **Batch 3** | 56.8 ± 4.5 | 97.9 ± 0.5 | $6.9x10^6$ | 5.1 ± 0.1 | 1500 | 0.60 – 0.87 |
| **Batch 4** | 57.0 ± 2.5 | 98.3 ± 0.2 | $6.6x10^6$ | 5.3 ± 0.2 | 1500 | 0.60 – 0.87 |
| **Batch 5** | 55.4 ± 3.1 | 96.9 ± 0.3 | $6.7x10^6$ | 5.5 ± 0.3 | 1500 | 0.58 – 0.84 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; sAF: specific airflow.

## 6.4. Results and discussion

This section is organised by contrasting each fermentation with its counterpart at 1.5 L and 22 L scales, to provide a straightforward comparison between scales in all tested strategies.

### 6.4.1. Batch strategy

#### 6.4.1.1. Rice husk scale-up

Figure 6.4 shows fermentation profiles in rice husk BB batch scaling; whereas Figure 6.6 shows same results for TH. Figures 6.5 and 6.7 show reactor appearance for BB and TH fermentations respectively.

Once stabilised, conidia production in all reactors with the same fungus was similar. For BB, both scales presented stabilised conidia production around $6.0x10^8$ conidia $g^{-1}$dm, although conidia production rise in 1.5 L was observed between 1-2 days later than in 22 L. For TH, both scales presented similar profiles, stabilising approximately at day 4 at values close to $1.6x10^9$ conidia $g^{-1}$dm. Both strains values were located in the range defined in Chapter 4. Despite its appearance in experiments summarized in Chapter 4 and its presence in rice husk, AN contamination was not detected in none of the batches.

In terms of biodegradability, no relevant differences were found between scales. Respiration profiles were similar, showing higher maximums at 22 L. Values at 1.5 L scale never surpassed 0.5 $gO_2$ $kg^{-1}$dm $h^{-1}$, whereas at 22 L rose up closer to 0.8-0.9 $gO_2$ $kg^{-1}$dm $h^{-1}$. This difference did not have a negative impact in heat generation, as mean temperature profiles were maintained below 30ºC in all fermentations. Observed behaviour confirms the feasibility of scaling-up fungal SSF fermentations using rice husk as substrate, as it presents no problems related to energy or mass transfer, at least to a scale of 22L. Rice husk advantages as substrate when scaling (naturally high $AFP_R$ and low biodegradability potential) were tested and confirmed as useful.

The rest of the parameters showed similar profiles within all reactors and strains, being similar to the ones shown in time course tests (Chapter 4), confirming the correct scaling of the process.

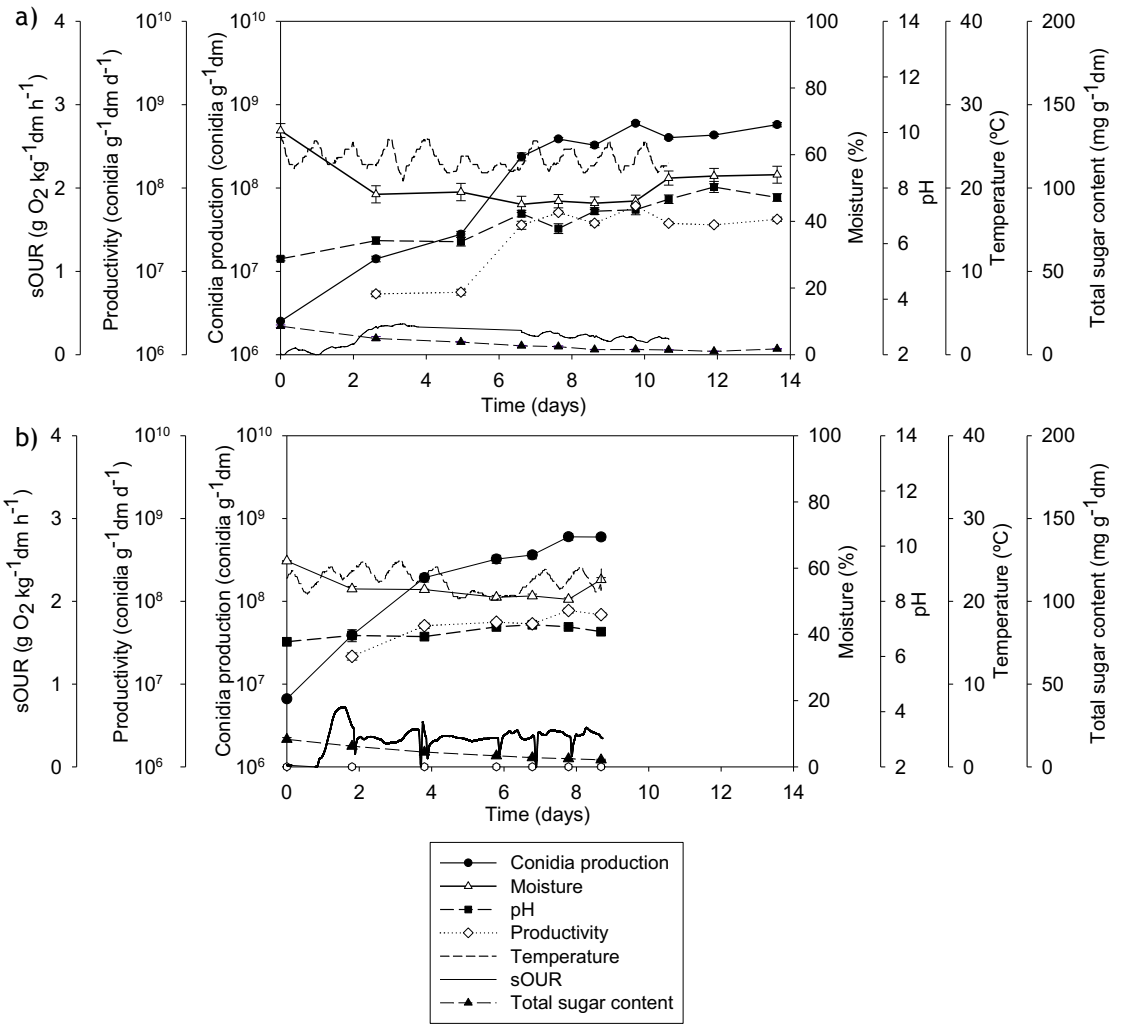



**Figure 6.4.** Process parameters evolution in rice husk BB batch strategy. a) 1.5 L and b) 22 L.

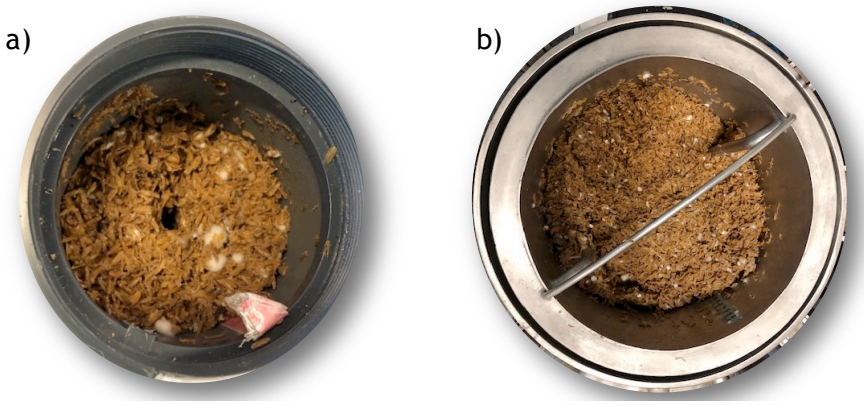



**Figure 6.5.** Reactor appearance in rice husk BB batch strategy. a) 1.5 L and b) 22 L.

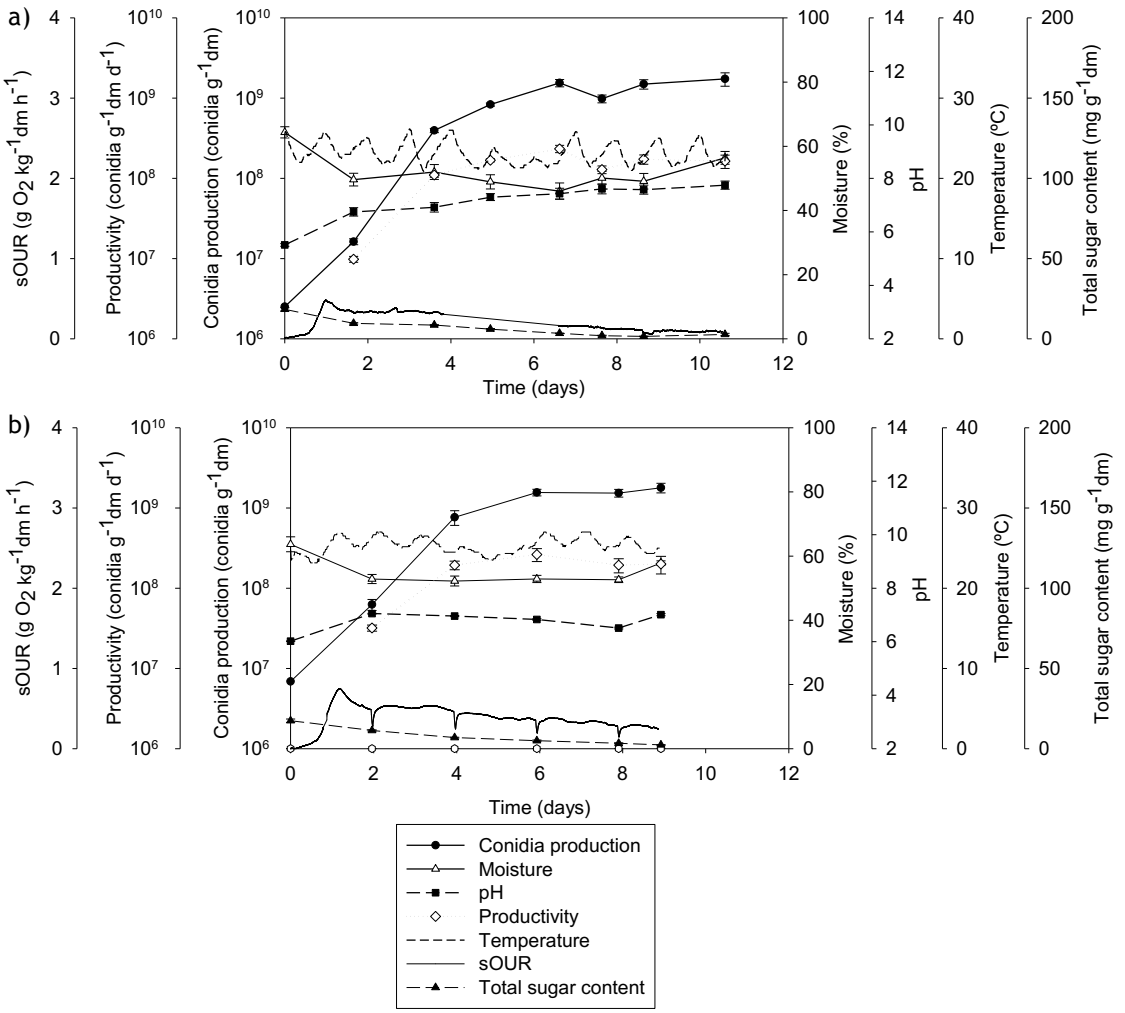



**Figure 6.6.** Process parameters evolution in rice husk TH batch strategy. a) 1.5 L and b) 22 L.

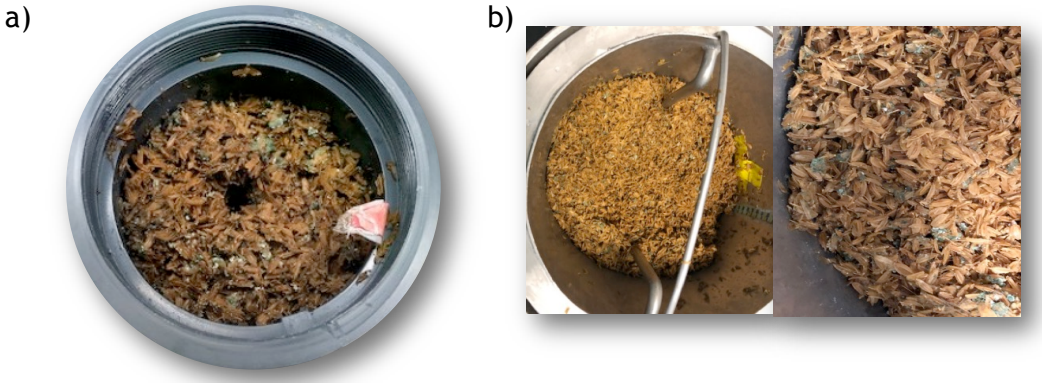



**Figure 6.7.** Reactor appearance in rice husk TH batch strategy. a) 1.5 L and b) 22 L.

Successful scaling of batch strategy using rice husk with both strains was achieved, serving as a first step before testing SBR strategy with this substrate.

### 6.4.1.2. Beer draff scale-up

Figure 6.8 shows obtained profiles in beer draff BB batch scaling; whereas Figure 6.10 shows same results for TH. Figures 6.9 and 6.11 show reactor appearance for BB and TH fermentations respectively.

Comparing to rice husk results, fungal fermentation scale-up presented some difficulties. Different combinations of substrate and bulking agent were tested (Tables 6.2 and 6.6). A 70/30 w/w (beer draff/wood chips) proportion was tested both at 1.5 L and 22 L scales using BB (figures 6.8 a) and b).). This mixture did not achieve BB conidia production in the 22 L bioreactor. Mean temperatures superior to 40ºC were achieved, while sOUR reached values close to 8 $gO_2$ $kg^{-1}$dm. This behaviour completely differed in comparison to any of the batches shown for both BB and TH, achieving much higher values both for temperature and sOUR. Adequate substrate-bulking agent mixture proportions for 1.5 L were not suitable for 22 L reactor due to substrate compaction, which highly reduces $AFP_R$ and oxygen transfer, subsequently difficulting fungal growth and sporulation and facilitating the appearance of contaminants (Krishna, 2005). $AFP_R$ adjustment (from values around 70 to values around 80) was shown as the key for the success of beer draff reactor scale-up. According to comparison between plates of non-inoculated samples of beer draff and wood chips separately (shown in Figure 6.12), contaminant in the second batch might have come from wood chips.

Conidia production obtained in BB batches was close to $1.5x10^9$ conidia $g^{-1}$dm at 1.5 L and of $2.5x10^9$ conidia $g^{-1}$dm at 22 L, while it was close to $2.0x10^9$ conidia $g^{-1}$dm for both TH scales. When working with BB, conidia production was vastly superior to the one achieved at 0.5 L scale in Chapter 5, where it was lower than $1.0x10^9$ conidia $g^{-1}$dm. Changes in substrate mixture resulting in higher $AFP_R$ values was the major cause of conidia production improvement. However, TH production decreased in comparison to the one achieved in Chapter 5, where it reached $7.5x10^9$ conidia $g^{-1}$dm. Although these changes are also due to substrate mixture, it would not be possible to perform the fermentation without the use of a bulking agent, as demonstrated in the failed BB batch. Consequently, conidia production diminishment was necessary for the correct scale-up of the process, as such high values might only be attainable when little to no bulking agent is needed to perform beer draff SSF, as it happened in Chapter 5.

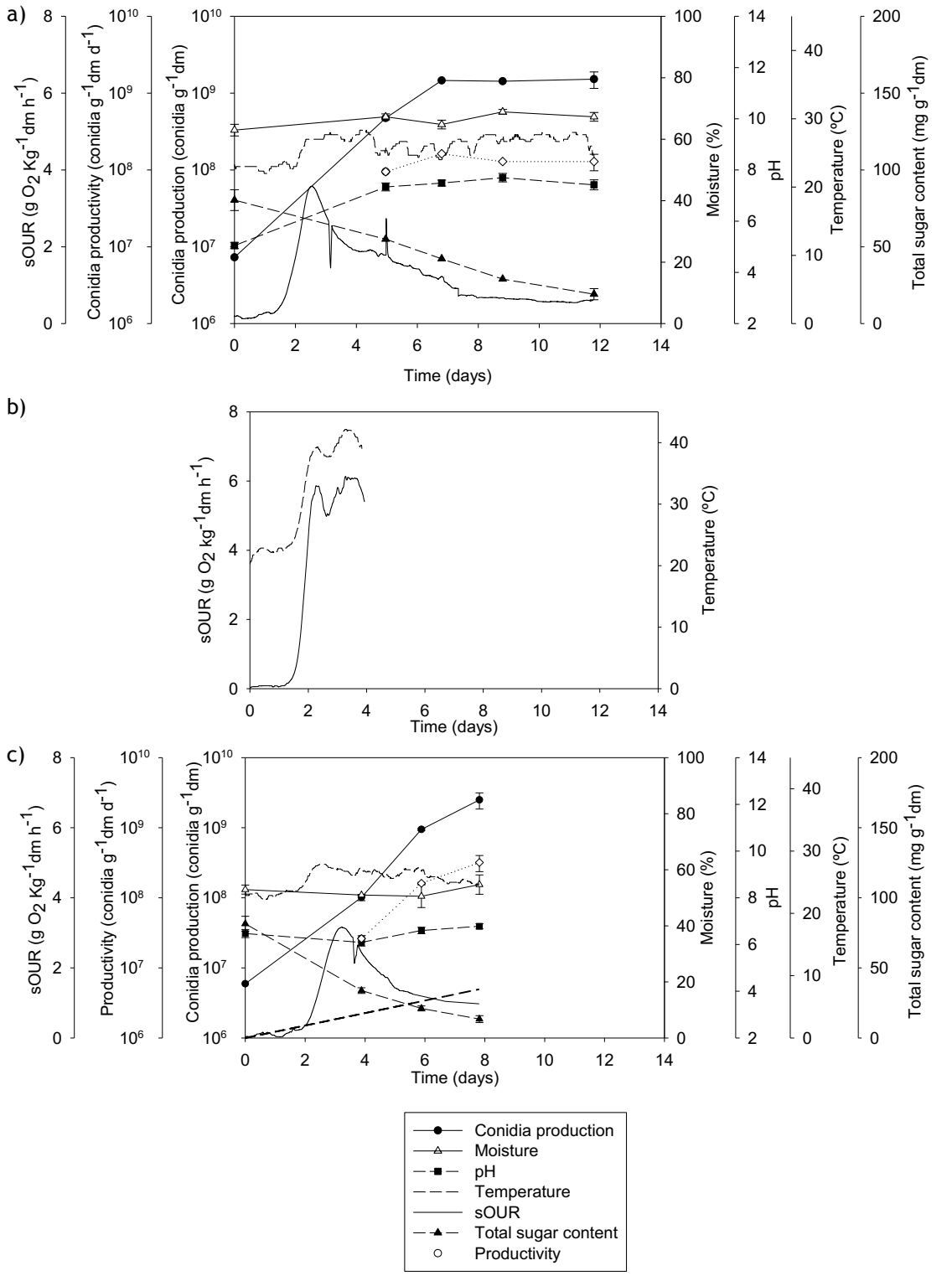



**Figure 6.8.** Process parameters evolution in beer draff BB batch strategy. a) 1.5 L, b) 22 L 70/30 w/w beer draff/wood chips and c) 22 L 40/60 w/w beer draff/wood chips.

In terms of biodegradability, differences between scales were minimal once $AFP_R$ was properly adjusted. All respiration profiles reached their maximum at values between 3-3.5 $gO_2$ $kg^{-1}$dm $h^{-1}$. Mean temperatures only surpassed 30ºC at both TH scales at the moment of maximum biological activity. Although mean temperature values did not rise due to scale-up, temperature differences between different areas of the reactor might be possible, especially at 22 L scale. Most SSF processes in packed beds present problems related to heat transfer and bed-packing at industrial or pilot scale, negatively affecting conidia production (Krishania et al., 2018). Due to its relevance, this effect will be examined in detail in the global analysis section (6.5).

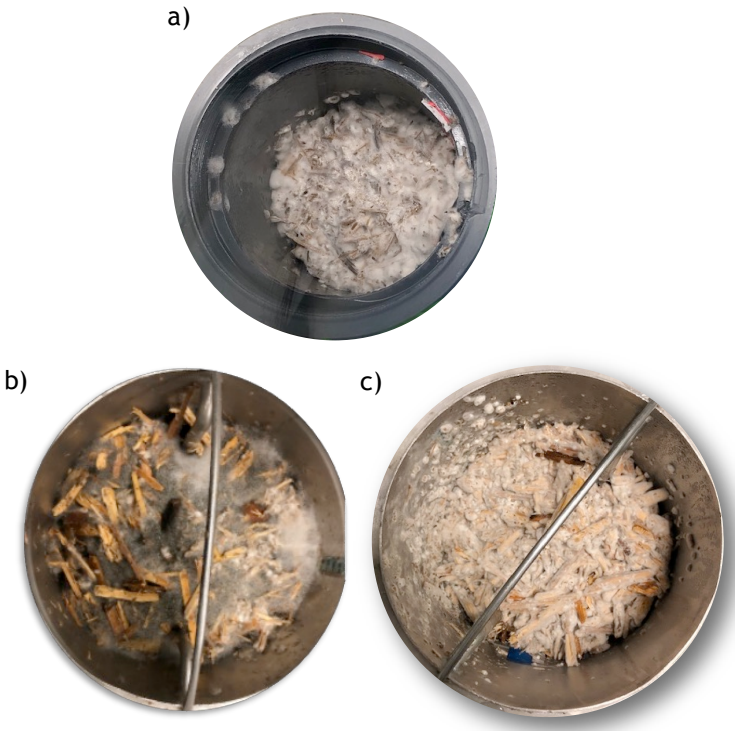



**Figure 6.9.** Reactor appearance in beer draff BB batch strategy. a) 1.5 L, b) 22 L 70/30 w/w beer draff/wood chips and c) 22 L 40/60 w/w beer draff/wood chips.

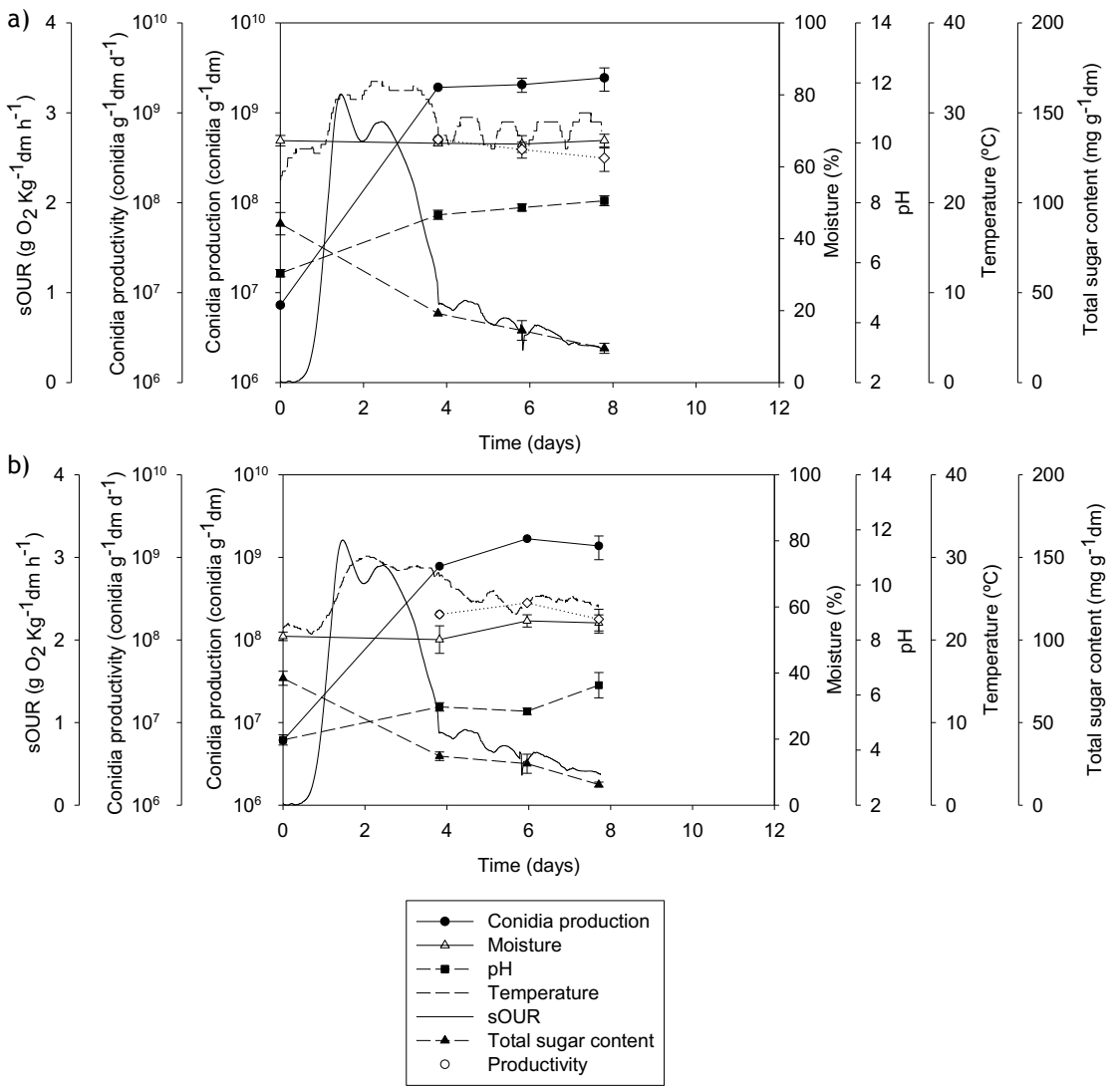



**Figure 6.10.** Process parameters evolution in beer draff TH batch strategy. a) 1.5 L and b) 22 L.

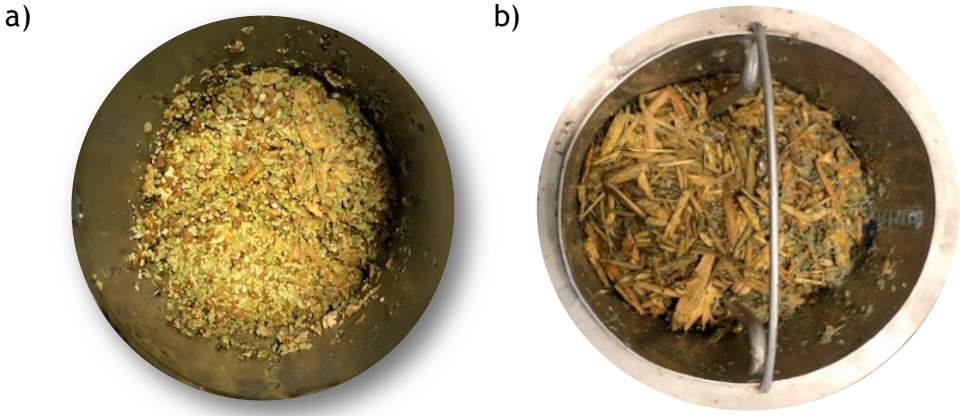



**Figure 6.11.** Reactor appearance in beer draff TH batch strategy. a) 1.5 L and b) 22 L.

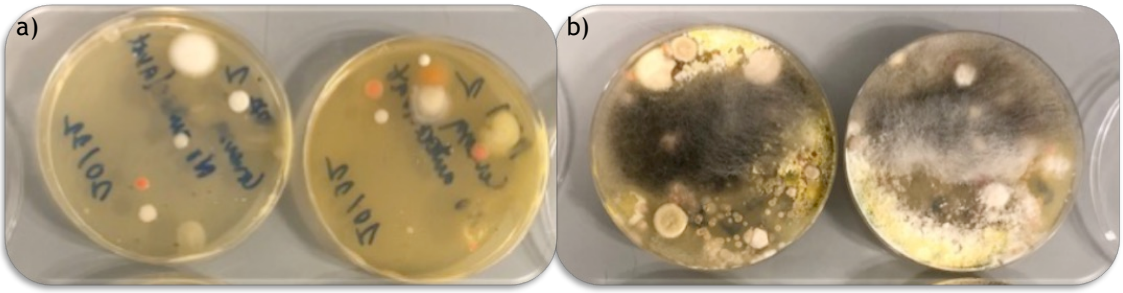



**Figure 6.12.** Plates inoculated with non-autoclaved extracts. a) Beer draff and b) wood chips.

Substrate mixture change also affected moisture and pH initial values. While in 1.5 L initial moisture was between 65-70% and initial pH was of 5.5-6, both values dropped in 22 L reactors to 50-55% moisture and 4-4.5 Better moisture adjustment for BB was achieved in 1.5 L, while it was better for TH in 22 L (optimums of 65-70% and 55-60% respectively, Chapter 4). As this pH values are not optimal for fungal growth and conidiation (Papagianni, 2004), initial pH was modified to values close to neutrality in BB fermentation. However, it was left untouched for TH, as some TH strains have been reported to work in acidic pH (Krishna, 2005; Zhang and Yang, 2015). Although both moisture and pH values had better adjustment for BB in 1.5 L batch, conidia production was superior in 22 L, highlighting $AFP_R$ relevance over other parameters.

After a proper $AFP_R$ adjustment, successful scaling of batch strategy using beer draff with both strains was achieved. These results serve as a first step before testing SBR strategy with this substrate, as well as confirming $AFP_R$ relevance in fungal SSF scale-up.

### 6.4.2. SBR strategy 1

SBR Strategy 1 results are presented in Figures 6.9 (BB) and 6.10 (TH). In both Figures, three different inoculum percentages for the SBR reactor are shown, being: a) 5%, b) 10% and c) 20%.

Important differences in behaviour were observed depending on the used strain. When working with BB, all SBR reactors presented AN contamination, showing no BB growth in the second reactor. However, this behaviour was not observed with TH, showing no AN contamination. As explained in previous Chapters, these differences might have been caused by TH's antifungal properties, which are not present in BB (Verma et al., 2007; Mascarin and Jaronski, 2016). Nevertheless, observed growth in all

TH SBR reactors was inferior when comparing with propagation reactors. In all propagation reactors, conidia production was 2 orders of magnitude higher in comparison to inoculum conidia concentration. However, it could not even rise one order of magnitude in all SBR reactors, with independence of the quantity of solid substrate used. This behaviour is reflected by conidia quotient values: whereas in propagation reactors it is of 54.6, it only rises to values close to 2.0 in all SBR reactors, showing irregular conidia distribution in all of them.

sOUR differences were observed between batches. Higher values were obtained in all BB SBR batches due to presence of contaminant AN. sOUR peaks also differed in time between batches, being of at least 2 days in the propagation reactors and always inferior (approx. 1.5 d) in all SBR reactors. Different behaviour between strains should be expected according to Aguilar-Zárate et al. (2018), as AN respiration peak would be located close to day 1 of fermentation, being always before BBs' peak. When working with TH, obtained sOUR peaks were similar in terms of maximum sOUR time in all batches. However, values were slightly higher in all SBR reactors, with the only exception of 20% SBR batch. This behaviour suggests 20% solid inoculum was a better strategy when compared to 5% and 10%, even though results were still not comparable to the ones obtained in the propagation reactors.

SBR Strategy 1 did not ensure a proper distribution of conidia throughout the new solid substrate, despite thoroughly mixing all the material before charging the SBR reactors. This behaviour could have been expected for BB, as it is normally used in form of diluted conidia suspension (Mascarin and Jaronski, 2016). However, results are more surprising for TH, as it is commonly used as biofertilizer or applying conidia within compost, with extraction from the solid not always being required (Verma et al., 2007; Bernal-Vicente et al., 2015). Mixing tests results in Chapter 4 also support BB difficulties to success with this strategy, as mixing negatively affected conidia production. However, they would suggest the possibility of obtaining better results using TH, as mixing was beneficial for conidia production when performed 24 or 48h after the start of the test.

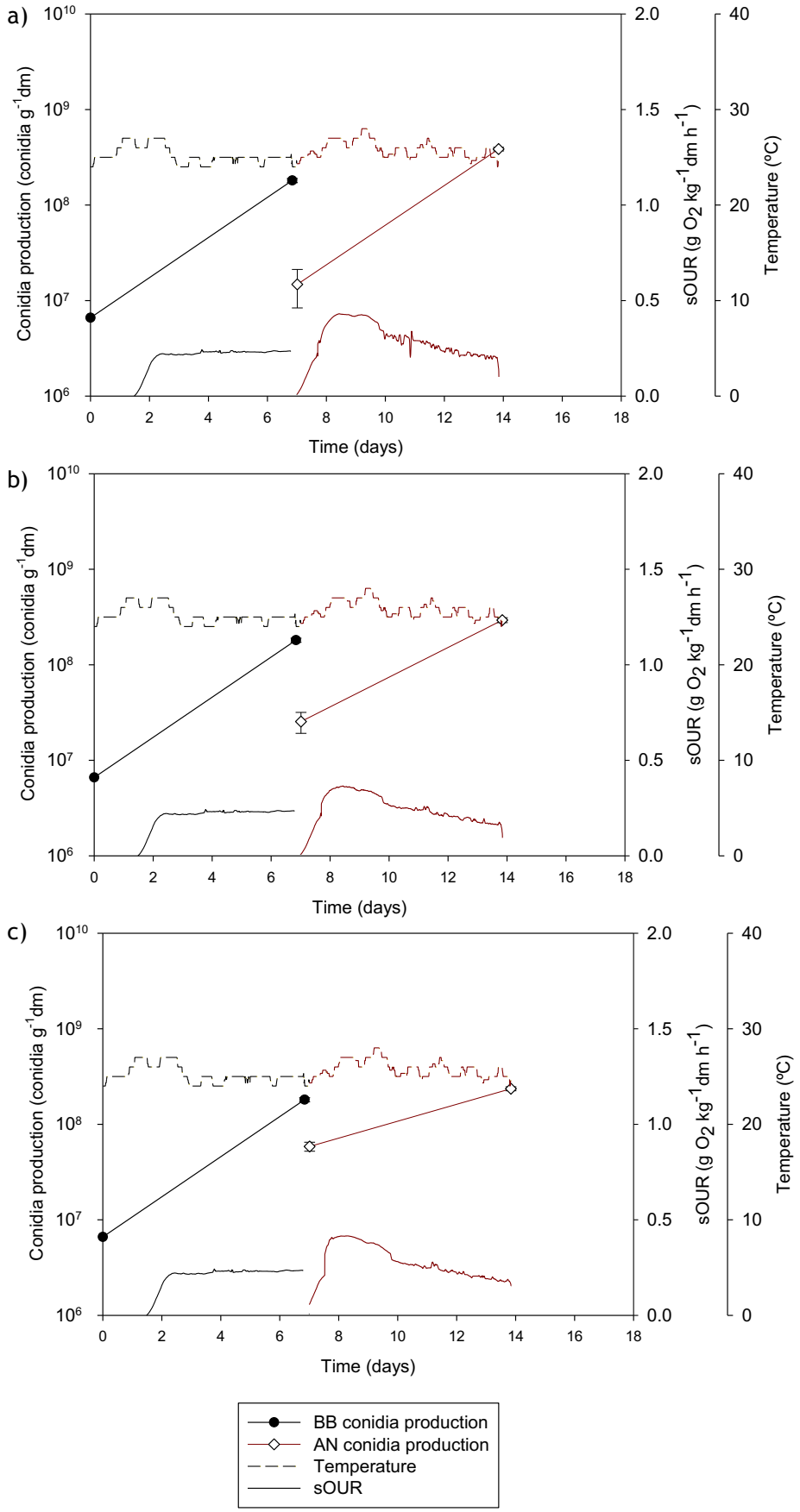



**Figure 6.13.** Process parameters evolution in 1.5 L BB sequential batch (strategy 1). a) 5% inoculum; b) 10% inoculum and c) 20% inoculum

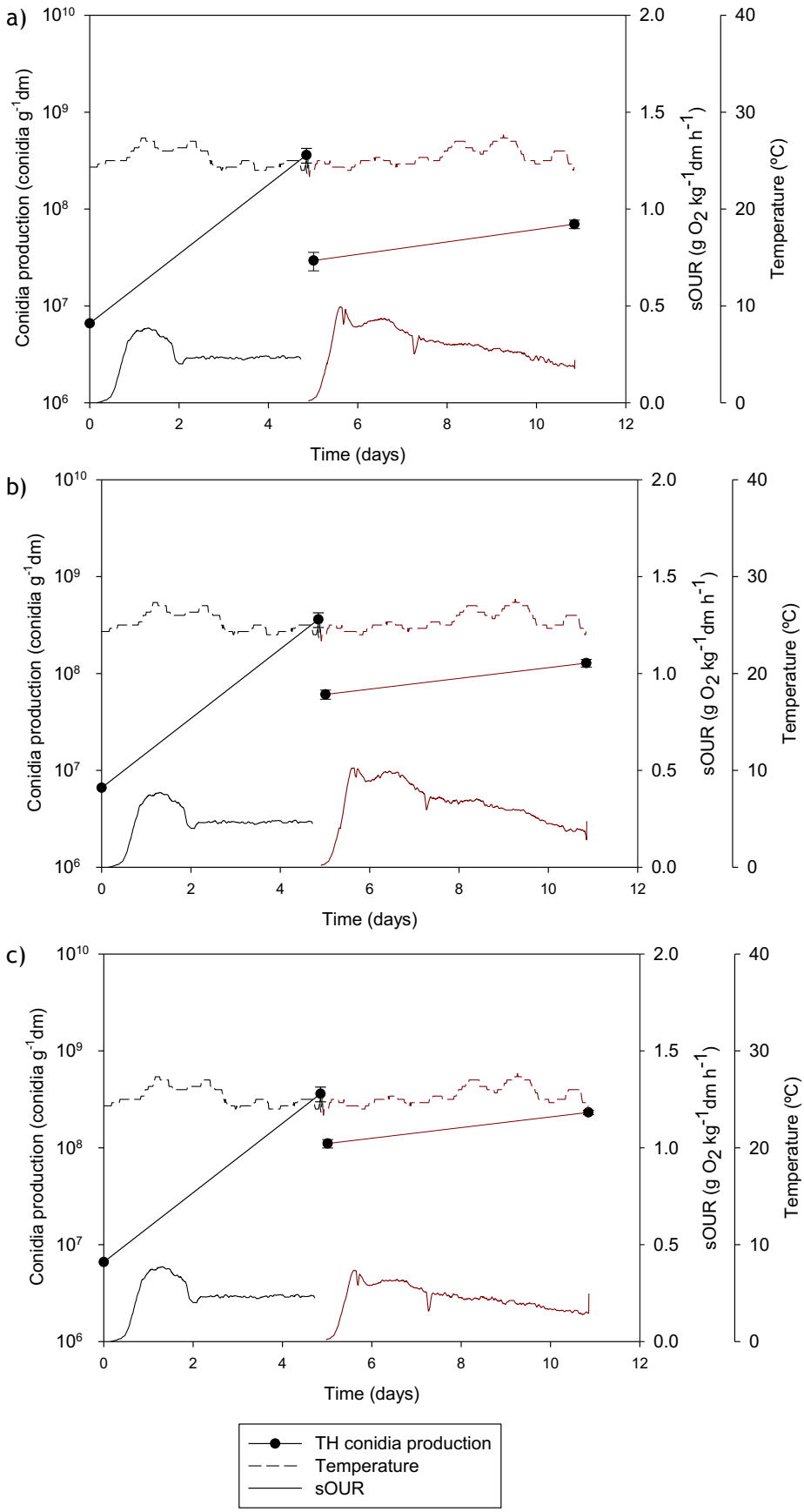



**Figure 6.14.** Process parameters evolution in 1.5 L TH sequential batch (strategy 1). a) 5% inoculum; b) 10% inoculum and c) 20% inoculum

### 6.4.3. SBR strategy 2

Moisture, pH and total sugar content ranges obtained in all SBR Strategy 2 fermentations are presented in Table 6.9. COC at 8 days (BB fermentations) and at 6 days (TH fermentations) is also shown.

#### 6.4.3.1. Inoculant age test

Figure 6.11 shows conidia productions using inoculum extracted from the fermentation reactor (rice husk) at days 4, 6, 8 and 9, both for BB (a) and TH (b). In BB test, all reactors were contaminated with AN, obtaining a co-culture of BB and AN. In TH test, no contamination was observed. In both tests, conidia concentration from the inoculum extracted at day 4 was significantly higher than the rest of the tested inoculant ages'. Although AN contaminant was detected in BB test, it was significantly different from BB concentration in day 4. Thus, 4 days was established as the optimum time for inoculum extraction for both BB and TH.

Inoculant age relevance has been highlighted for various fungal strains, as conidia quality tends to diminish with age. This is the case of Smith and Edgington (2011) using *Metarhizium* spp., Hallsworth and Magan (1996) using *Beauveria bassiana* and Múñiz-Paredes et al. (2016) using *Isaria fumosorosea*. However, and to our knowledge, this is the first time it has been tested using TH. Despite its relevance, inoculum age is not often studied in fungal growth optimization processes (Múñiz-Paredes et al., 2017).

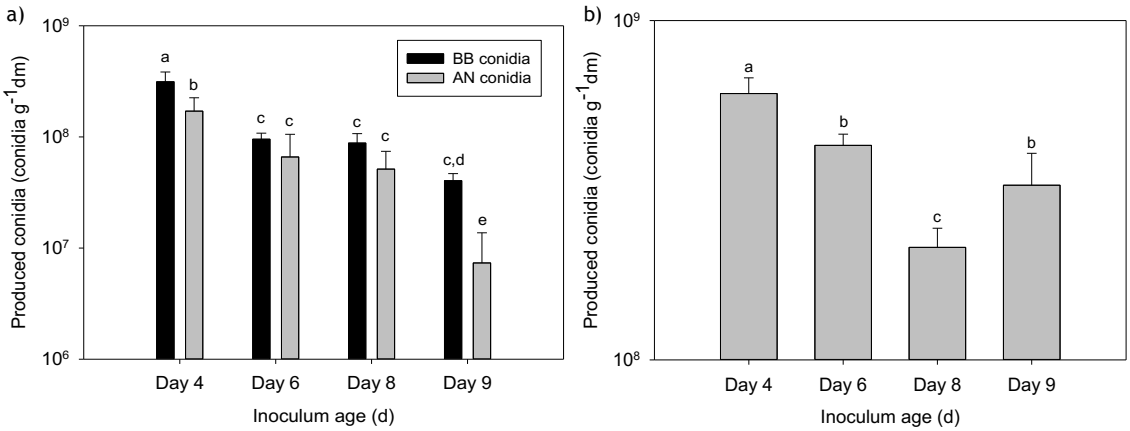



**Figure 6.15.** Conidia production in inoculant age tests. a) BB and b) TH.

### 6.4.3.2. Rice husk scale-up

Figure 6.16 shows obtained profiles in BB rice husk SBR operation. SBR strategy was only tested at 1.5 L scale.

Although propagation batch yielded almost $4.0x10^8$ conidia $g^{-1}dm$, BB conidia could not be counted in the SBR batch due to the presence of contaminant AN. Higher sOUR values (nearly doubling propagation reactor values) were achieved in the SBR batch (AN contaminated culture). After performing inoculant test and obtaining BB-AN co-culture in all second batches, AN presence in BB SBR batch was expected, however, also was BB conidia presence. As presented in Chapter 4, presence of AN contaminant in rice and its by-products is common (Aydin et al., 2011; Fredlund et al., 2009; Streit et al., 2012), while also being capable of withstanding autoclaving.

Use of a SBR strategy when working with rice husk would not be recommended due to AN contamination. For this reason, SBR strategy was not scaled-up using rice husk as substrate, concluding batch strategy as the preferable choice when working with BB and rice husk.

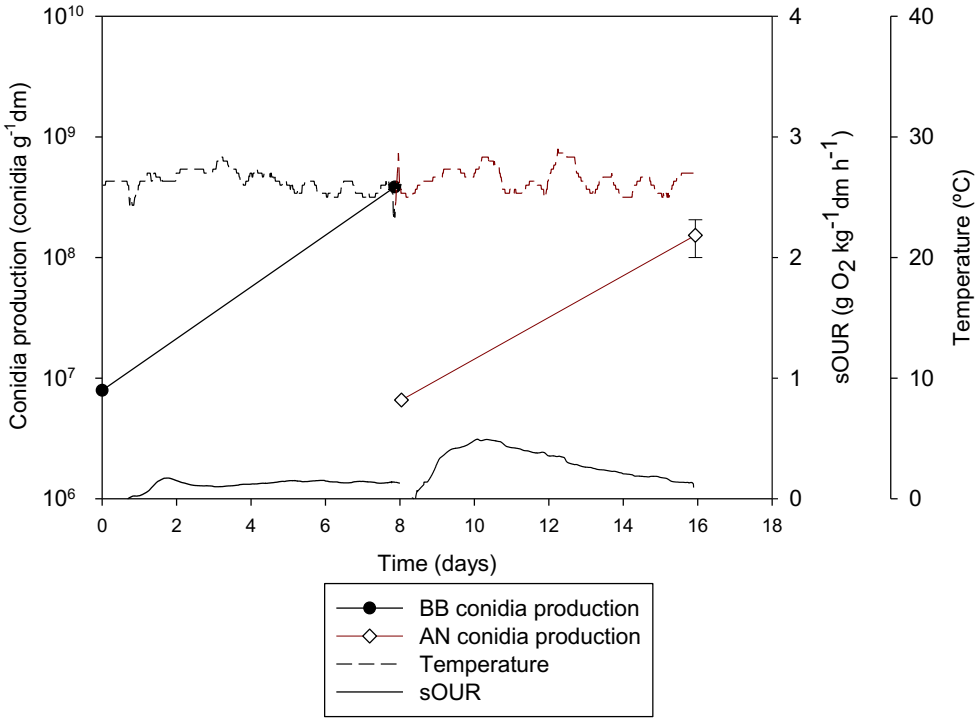



**Figure 6.16.** Process parameters evolution obtained in rice husk BB SBR strategy 2 scaling (1.5L reactors). Batch 1: black. Batch 2: brown.

Figure 6.17 shows obtained profiles in TH rice husk SBR operation, a) for 1.5 L and b) for 22 L.

At both scales, final conidia production on each reactor decreased halfway, starting around $1.4x10^9$ conidia $g^{-1}$dm in batch 1 and decreasing to values around $6.0x10^7$ conidia $g^{-1}$dm in batch 4 or 5. Maximum conidia production was achieved in day 6 of fermentation in all batches from 1 to 3, however, in batches 4 or 5 at both scales it was extended, achieving maximum conidia production in day 7. Conidia production reduction throughout the batches was apparently caused by the appearance of contaminant *Aspergillus niger* (AN) from batch 3 to batch 5. At both scales, AN conidia concentration doubled in each batch, starting at values close to $9.0x10^6$ conidia $g^{-1}$dm and rising to $8.3x10^7$ conidia $g^{-1}$dm in batch 5 in 1.5 L SBR, surpassing TH conidia production in the last batch. No fifth batch was performed at 22 L scale due to its behaviour being similar to 1.5 L.

Despite TH's antifungal properties (Verma et al., 2007), AN conidia were still able to grow in the substrate. Conidia production loss suggests AN growth started in the second batch, taking advantage of inoculum quality loss in comparison to pure inoculum extracted from plates used in the first batch, even though AN conidia could not be detected when counting due to their low numbers in comparison to TH conidia. With this result, production using rice husk at higher scales is still possible if working with single batch strategy using pure inoculum extracted from fresh plate.

In terms of biodegradability, respiration profiles were similar in all batches working at both scales, reaching maximum values close to 1 $gO_2$ $kg^{-1}$dm at similar times in most of the batches. These profiles are similar to those obtained in Chapter 4. Low potential biodegradability was also demonstrated with temperature profiles, as even at 22 L scale mean temperatures in the reactor never surpassed 32°C, starting approximately at 25°C in all fermentations. Higher temperature variation is to be expected when using substrates that present high or even moderate biodegradability, according to Barrena et al. (2011).

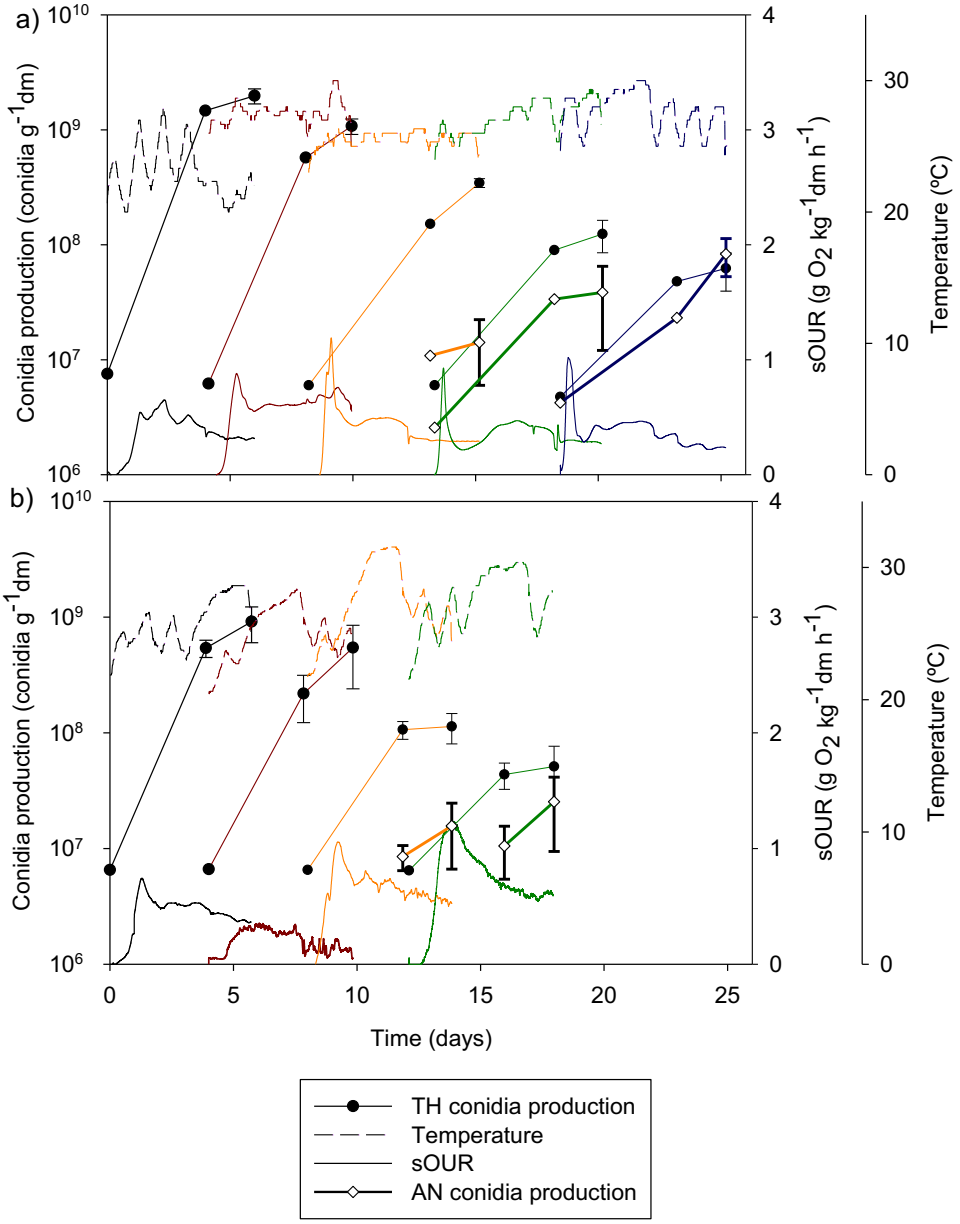



**Figure 6.17.** Process parameters evolution obtained in rice husk TH SBR strategy 2 scaling. a) 1.5 L and b) 22 L. Batch 1: black. Batch 2: brown. Batch 3: orange. Batch 4: green. Batch 5: blue.

Obtained values for moisture, pH and total sugar content were similar between scales: moisture ranged from 50-60% in most batches and pH started at values close to 6 and raised to values close to 7 at the end of all batches. Similar values for both parameters were observed at both scales. $AFP_R$ could easily be maintained at values able to ensure proper oxygen transfer at both scales (around 85%, as shown in Tables 6.7 and 6.8, being superior to the highest values of 80% indicated for the composting process by Ruggieri

et al. (2009)). Despite all parameters being adequate for TH growth and conidiation according to Chapter 4 results, co-culture growth was observed from batch 3 onwards.

Regarding AN growth parameters and according to several authors (Bhateria et al., 2019; Velasco-Alvarez et al., 2017), TH and AN co-culture growth was possible within the observed parameters' ranges, with both of them being present at least from batch 3 onwards. Although various studies using different AN strains have reported optimal acidic pH (3-6) for AN growth (Ghori et al., 2012; Passamani et al., 2014; Chang and Wang, 2018), neutral pH values of 7-8 did not diminish its presence. Moreover, AN growth over TH suggests faster growth of contaminant in rice husk in comparison to TH, promoting its prevalence in the co-culture in batch 5 in the 1.5 L SBR.

Rice husk has been found as an easy to scale-up substrate due to its naturally high porosity and low biodegradability, greatly reducing possible drawbacks caused by heat accumulation. However, despite successful scaling-up of the process (achieving similar results between both tested scales), presence of AN in the substrate suggests not to follow a SBR strategy when using rice husk. Consequently, a batch strategy using fresh inoculum appears to be the most optimal to maintain conidia concentration at its maximum, as best performances were obtained in the first batch (fresh inoculum) at both scales and could not be replicated in subsequent batches. As such, this strategy could be performed not only using rice husk as substrate but also when working with substrates which pose similar difficulties in terms of sterilization, which are common when working in SSF with agro-industrial wastes due to substrate heterogeneity (Yazid et al. (2017)).

#### 6.4.3.3. Beer draff scale-up

Figure 6.18 shows obtained profiles in BB beer draff SBR operation. SBR strategy was only tested at 1.5 L scale.

Comparing with same strategy using rice husk, BB growth and conidiation were observed in both reactors when using beer draff, obtaining a BB and AN co-culture in the SBR reactor. BB conidia reduction (from $1.3x10^9$ in the propagation reactor to $6.1x10^8$ in the SBR reactor) was observed. AN presence in SBR reactor was of $1.1x10^8$ conidia $g^{-1}$dm at day 10, corresponding to nearly 1/6 of the obtained BB conidia. BB growth was much slower in the SBR batch due to competition with AN. In almost 11 days of fermentation, conidia production did not show complete stabilization, being normally observed from day 6-7 onwards. As presented in the preliminary batch section, AN was

not present in the main substrate but in the bulking agent (wood chips), difficulting BB growth and colonization in the SBR batch.

Much as it happened with rice husk, use of a SBR strategy when working with beer draff would not be recommended due to AN contamination. Despite improvements when comparing to rice husk performance, SBR strategy was not scaled-up using beer draff as substrate, confirming batch strategy as the preferable choice when working with BB despite of the substrate used.

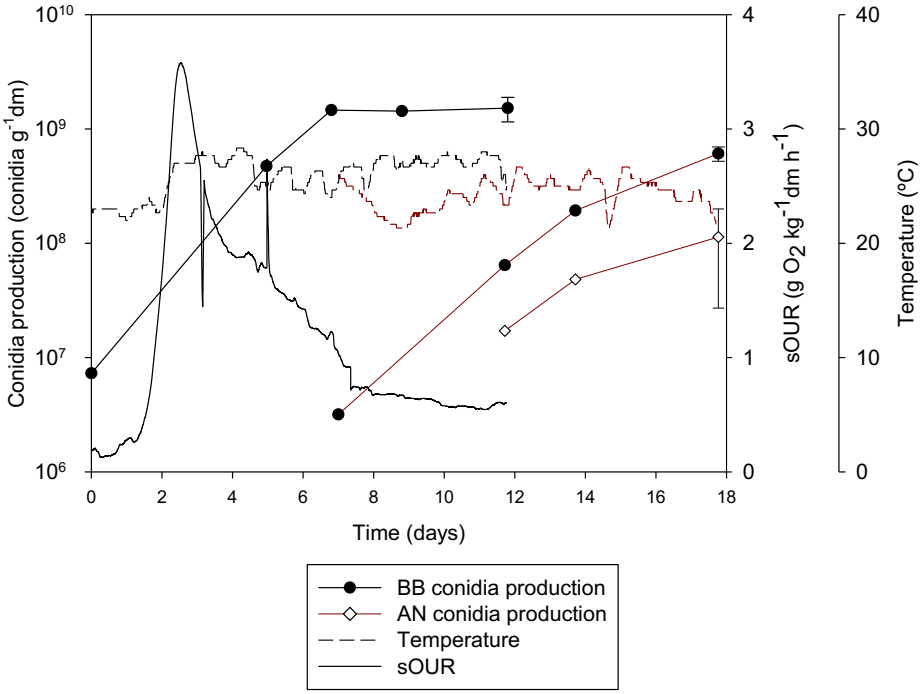



**Figure 6.18.** Process parameters evolution obtained in BB beer draff SBR strategy 2 scaling (1.5 L scale). Batch 1: black. Batch 2: brown.

Figure 6.19 shows obtained profiles in TH beer draff SBR operation, a) for 1.5 L and b) for 22 L. Figures 6.20 and 6.21 show reactor appearances for 1.5 and 22 L SBR respectively.

Contrarily to rice husk SBR results, different performances were observed comparing 1.5 L and 22 L SBR reactors. In 1.5 L reactors (Figure 6.15 a), conidia concentration was sustained for 3 consecutive batches, showing no significant differences among them, obtaining values between $1.6x10^9$ conidia $g^{-1}$dm and $2.1x10^9$ conidia $g^{-1}$dm. However, batch 4 yielded a significantly lower conidia production ($5.6x10^8$ conidia $g^{-1}$dm). Maximum conidia production was achieved in day 6 of fermentation in batches 1 and 2; however, in batches 3 and 4 fermentation time was extended and maximum conidia

production was achieved in day 8. In contrast, conidia concentrations at 22 L (Figure 6.15 b) was sustained in all batches, obtaining values between $1.7x10^9$ conidia $g^{-1}$dm and $2.2x10^9$ conidia $g^{-1}$dm, showing no significant differences between all 5 batches. Maximum conidia concentration was achieved within 6 days in 4 out of 5 batches, while in batch 5, 8 days where needed. Longer time might have been due to differences in temperature profiles between batch 5 and the rest of the SBR batches. AN contamination was not detected at any scale, confirming the substrate change as a valid decision.

Differences in performance between SBR at 1.5 L and SBR at 22 L could be attributable to the combined effects of three parameters: $AFP_R$, pH and moisture. As presented in Tables 6.5 and 6.8, initial $AFP_R$ was different between scales: 72.6 in 1.5 L vs 81.2 in 22 L. $AFP_R$ adjustment was key for the success of the SBR strategy, as 22 L SBR had 5 consecutive batches which presented the same behaviour, with the possibility of lengthening the process even more, while 1.5 L SBR only had 3. pH variation between scales was similar to values presented in the preliminary batches, obtaining more acidic values in the 22 L fermentations. Acidic values might have been favourable for TH, as previously seen in the 22 L preliminary batch. Moisture behaviour was also similar, showing lower range in 22 L batches.

In terms of biodegradability, all obtained respiration profiles were similar, reaching maximum values close to 3.5 $gO_2$ $kg^{-1}$dm $h^{-1}$ at comparable times in nearly all of the batches, with the only exception of batch 5 in 22 L SBR, which only reached 2.5 $gO_2$ $kg^{-1}$dm $h^{-1}$. Much higher total sugars available (80-88 mg $g^{-1}$dm) combined with higher values of IRD, sOUR and $COC_{6d}$ also indicated higher substrate biodegradability when comparing to rice husk. Mean temperatures in batch 5 were lower than those obtained in the rest of batches, being even lower than 20ºC at the beginning of the fermentation. Due to the relevance of temperature in fungal growth (Hallsworth and Magan, 1996), lower values of this parameter might be the cause of fungal growth and conidia production lengthening time in batch 5. Even though mean temperature profiles at both scales did not highly differ from profiles obtained when working with rice husk, using a substrate with higher potential biodegradability caused temperature gradients in the reactor. Observed differences in batch 5 in 22 L SBR were probably caused by non-optimal range values in the analysed parameters rather than lower inoculum quality, as might have happened at 1.5 L scale. Optimal values for all process parameters will be discussed in the global analysis section.

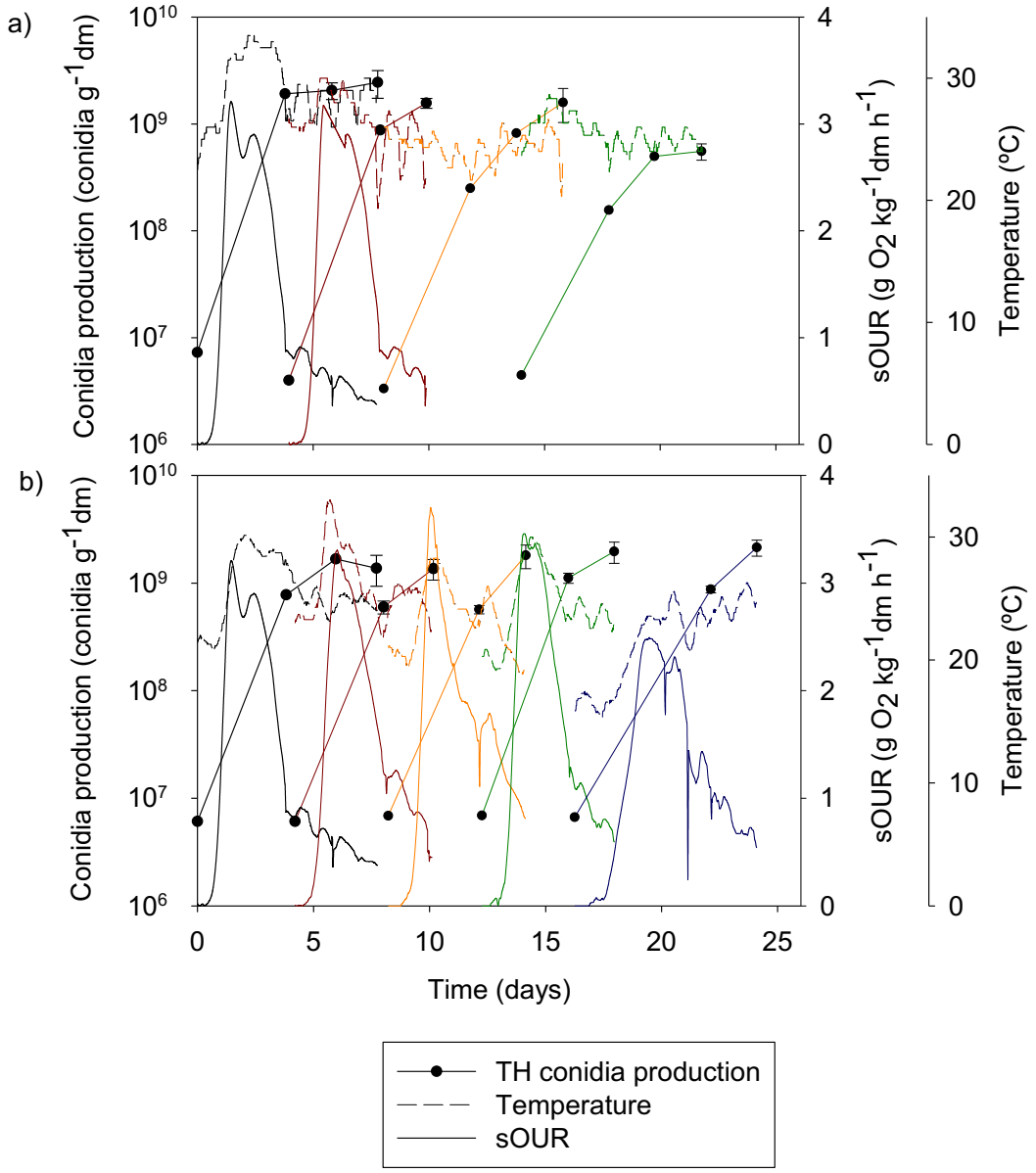



**Figure 6.19.** Process parameters evolution obtained in beer draff TH SBR strategy 2 scaling. a) 1.5 L and b) 22 L Batch 1: black. Batch 2: brown. Batch 3: orange. Batch 4: green. Batch 5: blue.

Successful scaling from 1.5 L to 22 L has been achieved using beer draff as substrate and wood chips as bulking agent in a SBR operation. However, SBR performance differed between scales: while at 1.5 L conidia concentration decreased from batch 4 onwards due to loss of inoculum quality, at 22 L scale 5 batches were performed achieving similar maximum conidia concentrations. Differences between the two scales have been mainly caused by the use of different substrate-bulking agent mixtures, being 70-30% in 1.5 L and 40-60% in 22 L. These results suggest that a minimum $AFP_R$ value of around 80% is needed to ensure proper fungal growth when working in packed bed

reactors, highlighting the need to find the optimal $AFP_R$ value when scaling up SSF fungal conidia production processes operating with and SBR strategy. Particle size relevance in SSF studies has been highlighted by Yazid et al. (2017). Small particle size provides larger surface area for fungal growth while being prone to agglomeration and difficulties in oxygen transfer. In contrast, large particle size provides better oxygen transfer and reduces heat accumulation at the cost of limiting surface growth area. Higher surface area helps at maximizing mycelial growth, which is necessary for correct fungal sporulation. Additionally, mycelial growth does not affect substrate porosity, meaning it should be maximized before conidiation (Casciatori et al., 2014). Balance between different particle sizes in large reactors is mandatory to ensure proper fungal growth and sporulation. These findings are highly relevant to fungal SSF, as they establish a reproducible method to overcome SSF traditional drawbacks while defining biodegradability and $AFP_R$ as the key parameters in SSF scale–up. The results presented open the possibility of performing the current process using packed beds at higher scales.


**Figure 6.20.** Reactor appearance of 1.5 L TH beer draff SBR fermentations.

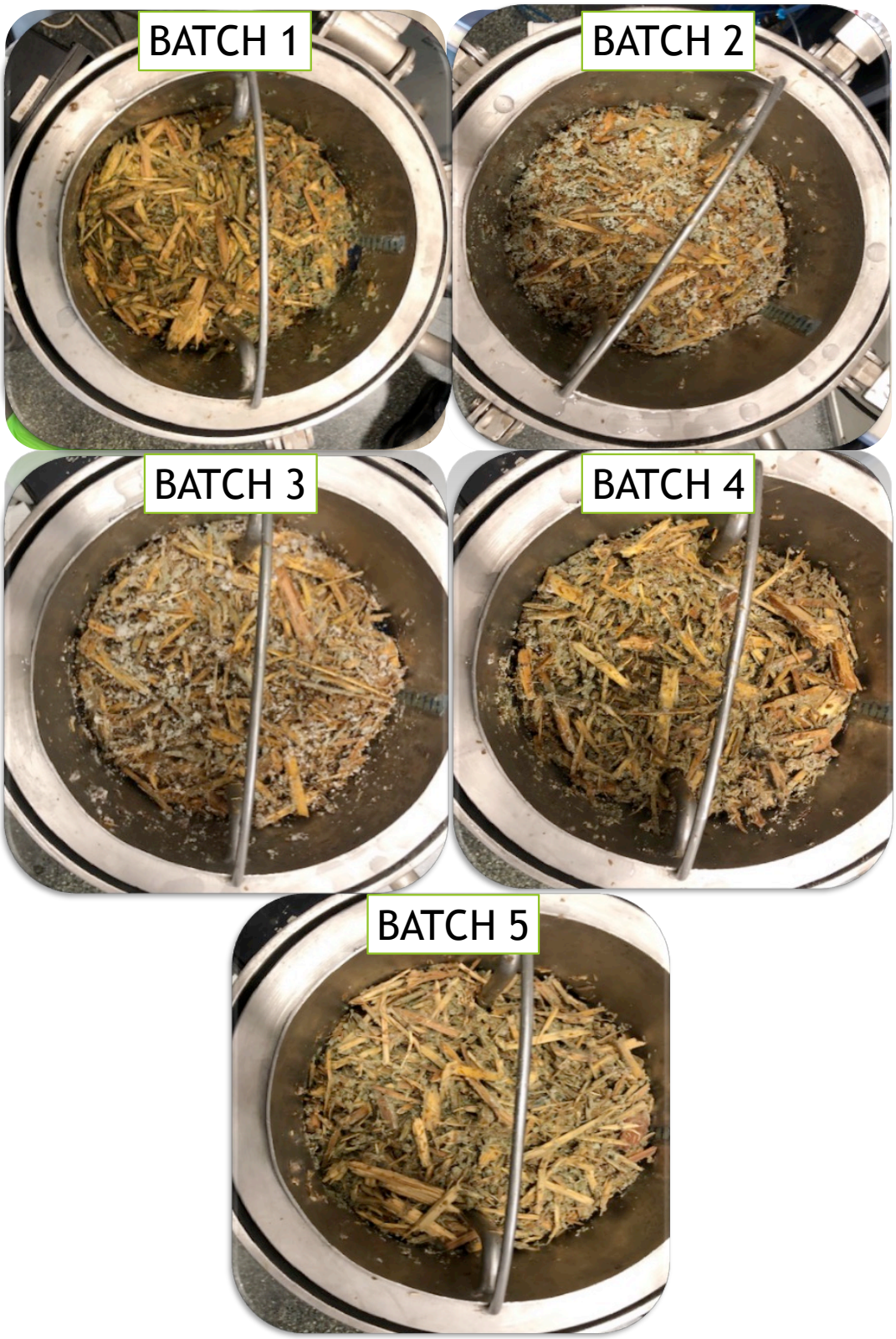



**Figure 6.21.** Reactor appearance of 22 L TH beer draff SBR fermentations.

**Table 6.9.** Moisture, pH and total sugar content ranges in all strategy 2 SBR batches. Initial and final values are shown. Cumulative oxygen consumption at day 8 for BB fermentations and at day 6 for TH fermentations is also shown.

| Batch/parameter | Moisture range (%) | pH range | Total sugar content range (mg $g^{-1}$dm) | Cumulative oxygen consumption at day 8 (BB) or 6 (TH) ($gO_2$ $kg^{-1}$dm) |
|---|---|---|---|---|
| 1.5 L BB rice husk Batch 1 | 62.5±1.0 – 51.4±0.8 | 5.6±0.2 – 7.0±0.3 | 13.7±0.5 – 5.5±0.3 | 1.2 |
| 1.5 L BB rice husk Batch 2 | 69.7±1.4 – 58.4±1.1 | 5.7±0.1 – 7.0±0.2 | 13.1±0.7 – 2.9±0.3 | 3.0 |
| 1.5 L TH rice husk Batch 1 | 64.2±1.0 – 61.0±1.0 | 6.1±0.1 – 6.9±0.1 | 12.4±0.8 – 3.3±0.4 | 1.8 |
| 1.5 L TH rice husk Batch 2 | 56.4±1.1 – 55.8±1.0 | 6.3±0.2 – 6.5±0.3 | 11.9±0.6 – 3.0±0.2 | 2.1 |
| 1.5 L TH rice husk Batch 3 | 54.9±0.9 – 50.0±0.7 | 6.6±0.2 – 7.2±0.2 | 13.1±0.6 – 2.9±0.2 | 1.7 |
| 1.5 L TH rice husk Batch 4 | 55.0±1.0 – 57.4±1.8 | 6.6±0.2 – 6.9±0.2 | 14.4±0.5 – 2.8±0.2 | 1.6 |
| 1.5 L TH rice husk Batch 5 | 57.8±1.3 – 61.1±1.5 | 6.6±0.2 – 7.2±0.2 | 13.9±0.5 – 3.0±0.2 | 1.6 |

**Table 6.9. (cont).** Moisture, pH and total sugar content ranges in all strategy 2 SBR batches. Initial and final values are shown. Cumulative oxygen consumption at day 8 for BB fermentations and at day 6 for TH fermentations is also shown.

| Batch/parameter | Moisture range (%) | pH range | Total sugar content range (mg $g^{-1}$dm) | Cumulative oxygen consumption at day 8 (BB) or 6 (TH) ($gO_2$ $kg^{-1}$dm) |
|---|---|---|---|---|
| 22 L TH rice husk Batch 1 | 58.7±0.3 – 47.7±0.8 | 6.6±0.1 – 7.3±0.1 | 13.6±0.8 – 3.2±0.4 | 1.8 |
| 22 L TH rice husk Batch 2 | 60.4±1.9 – 54.0±1.2 | 6.9±0.2 – 7.2±0.1 | 13.0±0.9 – 3.0±0.4 | 1.1 |
| 22 L TH rice husk Batch 3 | 57.5±1.1 – 57.6±1.2 | 7.0±0.1 – 7.0±0.2 | 14.0±0.9 – 3.0±0.3 | 2.2 |
| 22 L TH rice husk Batch 4 | 58.2±1.2 – 60.1±1.2 | 6.7±0.2 – 7.3±0.2 | 13.3±1.1 – 2.7±0.3 | 2.6 |
| 1.5 L BB beer draff Batch 1 | 63.0±1.9 – 67.3±1.4 | 5.1±0.1 – 7.4±0.2 | 80.4±6.7 – 19.2±3.6 | 13.3 |
| 1.5 L BB beer draff Batch 2 | 59.2±0.8 – 58.9±1.7 | 5.6±0.1 – 7.3±0.1 | 83.4±7.0 – 14.9±1.6 | - |
| 1.5 L TH beer draff Batch 1 | 67.3±1.5 – 67.3±1.8 | 5.7±0.1 – 8.1±0.2 | 88.4±6.2 – 19.1±2.8 | 17.8 |
| 1.5 L TH beer draff Batch 2 | 67.4±0.3 – 67.0±2.5 | 5.9±0.2 – 7.8±0.5 | 84.2±6.8 – 16.9±2.5 | 17.4 |
| 1.5 L TH beer draff Batch 3 | 61.2±1.3 – 60.0±1.7 | 5.5±0.1 – 6.9±0.3 | 81.4±6.8 – 13.1±3.1 | - |
| 1.5 L TH beer draff Batch 4 | 61.2±0.5 – 65.0±3.2 | 5.7±0.2 – 7.3±0.1 | 83.7±5.2 – 11.0±3.1 | - |

**Table 6.9. (cont).** Moisture, pH and total sugar content ranges in all strategy 2 SBR batches. Initial and final values are shown. Cumulative oxygen consumption at day 8 for BB fermentations and at day 6 for TH fermentations is also shown.

| Batch/parameter | Moisture range (%) | pH range | Total sugar content range (mg $g^{-1}$dm) | Cumulative oxygen consumption at day 6 ($gO_2$ $kg^{-1}$dm) |
|---|---|---|---|---|
| 22 L beer draff Batch 1 | 51.1±1.3 – 55.2±2.4 | 4.5±0.2 – 6.4±0.5 | 76.8±4.3 – 12.5±1.5 | 18.2 |
| 22 L beer draff Batch 2 | 55.9±3.1 – 58.2±2.6 | 4.8±0.2 – 5.6±0.2 | 88.4±6.2 – 29.0±5.4 | 17.5 |
| 22 L beer draff Batch 3 | 56.8±4.5 – 58.4±2.2 | 5.1±0.2 – 5.1±0.3 | 75.2±3.8 – 11.2±2.0 | 19.1 |
| 22 L beer draff Batch 4 | 57.1±2.5 – 57.5±1.8 | 5.3±0.2 – 5.0±0.3 | 83.1±5.4 – 21.3±3.7 | 18.8 |
| 22 L beer draff Batch 5 | 55.4±3.1 – 57.2±3.2 | 5.5±0.2 – 5.3±0.3 | 79.5±5.0 – 17.1±2.4 | 15.6 |

BB: *Beauveria bassiana*; TH: *Trichoderma harzianum*.

**Table 6.10.** Summarized conidia production results obtained in the process scale-up (produced conidia, productivity and conidia quotient). Only preliminary and SBR Strategy 2 batches are presented.

| Test | Substrate | Reactor | Produced conidia (conidia $g^{-1}dm$) | Productivity (conidia $g^{-1}dm\ d^{-1}$) | CQ |
|---|---|---|---|---|---|
| Preliminary batches | Rice husk | 1.5 L BB | $6.0x10^{8}$ | $8.6x10^{7}$ | 119 |
| | | 1.5 L TH | $1.5x10^{9}$ | $2.4x10^{8}$ | 261 |
| | | 22 L BB | $6.0x10^{8}$ | $7.7x10^{7}$ | 107 |
| | | 22 L TH | $1.6x10^{9}$ | $2.6x10^{8}$ | 284 |
| | Beer draff | 1.5 L BB | $1.5x10^{9}$ | $2.3x10^{8}$ | 192 |
| | | 1.5 L TH | $1.8x10^{9}$ | $2.9x10^{8}$ | 242 |
| | | 22 L BB* | $2.5x10^{9}$ | $3.2x10^{8}$ | 402 |
| | | 22 L TH | $1.9x10^{9}$ | $2.9x10^{8}$ | 274 |
| SBR Strategy 2 | Rice husk | 1.5 L BB propag. | $3.8x10^{8}$ | $4.9x10^{7}$ | 58.2 |
| | | 1.5 L BB SBR** | **(-) | **(-) | **(-) |
| | | 1.5 L TH B1 | $2.0x10^{9}$ | $3.3x10^{8}$ | 300 |
| | | 1.5 L TH B2 | $1.1x10^{9}$ | $2.0x10^{8}$ | 163 |
| | | 1.5 L TH B3** | $3.5x10^{8}$** | $5.0x10^{7}$** | 52** |
| | | 1.5 L TH B4** | $1.3x10^{8}$** | $2.0x10^{7}$** | 19** |
| | | 1.5 L TH B5** | $6.2x10^{8}$** | $9.8x10^{6}$** | 9** |

**Table 6.10. (cont).** Summarized conidia production results obtained in the process scale-up (produced conidia, productivity and conidia quotient). Only preliminary and SBR Strategy 2 batches are presented.

| Test | Substrate | Reactor | Produced conidia (conidia $g^{-1}dm$) | Productivity (conidia $g^{-1}dm\ d^{-1}$) | CQ |
|---|---|---|---|---|---|
| SBR Strategy 2 | Rice husk | 22 L TH B1 | $9.1x10^8$ | $1.6x10^8$ | 152 |
| | | 22 L TH B2 | $5.4x10^8$ | $9.5x10^7$ | 90 |
| | | 22 L TH B3** | $1.1x10^8$** | $2.0x10^7$** | 18** |
| | | 22 L TH B4** | $6.3x10^7$** | $1.0x10^7$** | 11** |
| | Beer draff | 1.5 L BB propag. | $1.3x10^9$ | $2.2x10^8$ | 115 |
| | | 1.5 L BB SBR** | $6.1x10^8$** | $4.8x10^7$** | 57** |
| | | 1.5 L TH B1 | $2.1x10^9$ | $4.0x10^8$ | 206 |
| | | 1.5 L TH B2 | $1.6x10^9$ | $2.6x10^8$ | 262 |
| | | 1.5 L TH B3 | $1.6x10^9$ | $1.7x10^8$ | 273 |
| | | 1.5 L TH B4 | $5.6x10^8$ | $7.8x10^7$ | 70 |
| | | 22 L TH B1 | $1.7x10^9$ | $2.8x10^8$ | 254 |
| | | 22 L TH B2 | $1.4x10^9$ | $2.3x10^8$ | 207 |
| | | 22 L TH B3 | $1.8x10^9$ | $3.0x10^8$ | 274 |
| | | 22 L TH B4 | $2.0x10^9$ | $3.4x10^8$ | 298 |
| | | 22 L TH B5 | $2.2x10^9$ | $2.7x10^8$ | 325 |

CQ: conidia quotient; *Successful 22 L preliminary batch; BB: *Beauveria bassiana*; TH: *Trichoderma harzianum*;**: detected AN contamination; propag: propagation reactor; SBR: sequential batch reactor; B: batch.

## 6.5. Global analysis

To test the robustness and reproducibility for fungal conidia production in packed bed reactor operating under a SBR strategy, statistical analyses were performed using data collected from sampling of the final solid material of each 22 L batch with both substrates. Preliminary batches were used for BB data with both substrates, while all SBR TH batches data was used (4 batches for rice husk and 5 for beer draff).

Figures 6.22 (BB) and 6.23 (TH) show mean values and standard deviations obtained when analysing samples at the end of each batch depending on their height in the reactor. Results are shown for conidia production, moisture and pH in all 22 L batches. When comparing process parameters within the same batch, little quantity of significant differences is shown thorough the packed bed for both substrates, while no patterns are followed in terms of maximum conidia production height in the reactor. It can be assumed that conidia production does not depend on bed height in a 22 L rice husk or beer draff packed bed reactor, even though it could still be dependent on bed height at higher scales. This is consequent with little moisture and pH variations shown in all batches, with nearly all the compared samples not presenting significant differences with the rest of the samples in the same batch. These data highlight the robustness of the process, as both tested substrates packed beds had similar physical properties at all heights.

When working with TH, pH differences between substrates are clearly shown. Lower pH values were achieved with beer draff in comparison to rice husk and corresponding to the batches which yielded highest conidia productions, meaning acidic pH might have been beneficial for TH growth and conidiation. These results differ from the majority of the references in bibliography, as TH growth and conidiation optimums are normally located near neutral pH, even though TH can grow and sporulate within an initial pH range of 3-9 (Zhang and Yang, 2015; Papagianni, 2004). These differences in behaviour when comparing with literature could have been caused by using a specific *Trichoderma* strain which could present better results when working at acidic pH. Some *Trichoderma* spp. strains have been demonstrated to work optimally under acidic pH conditions (Hong-Jun et al., 2021).

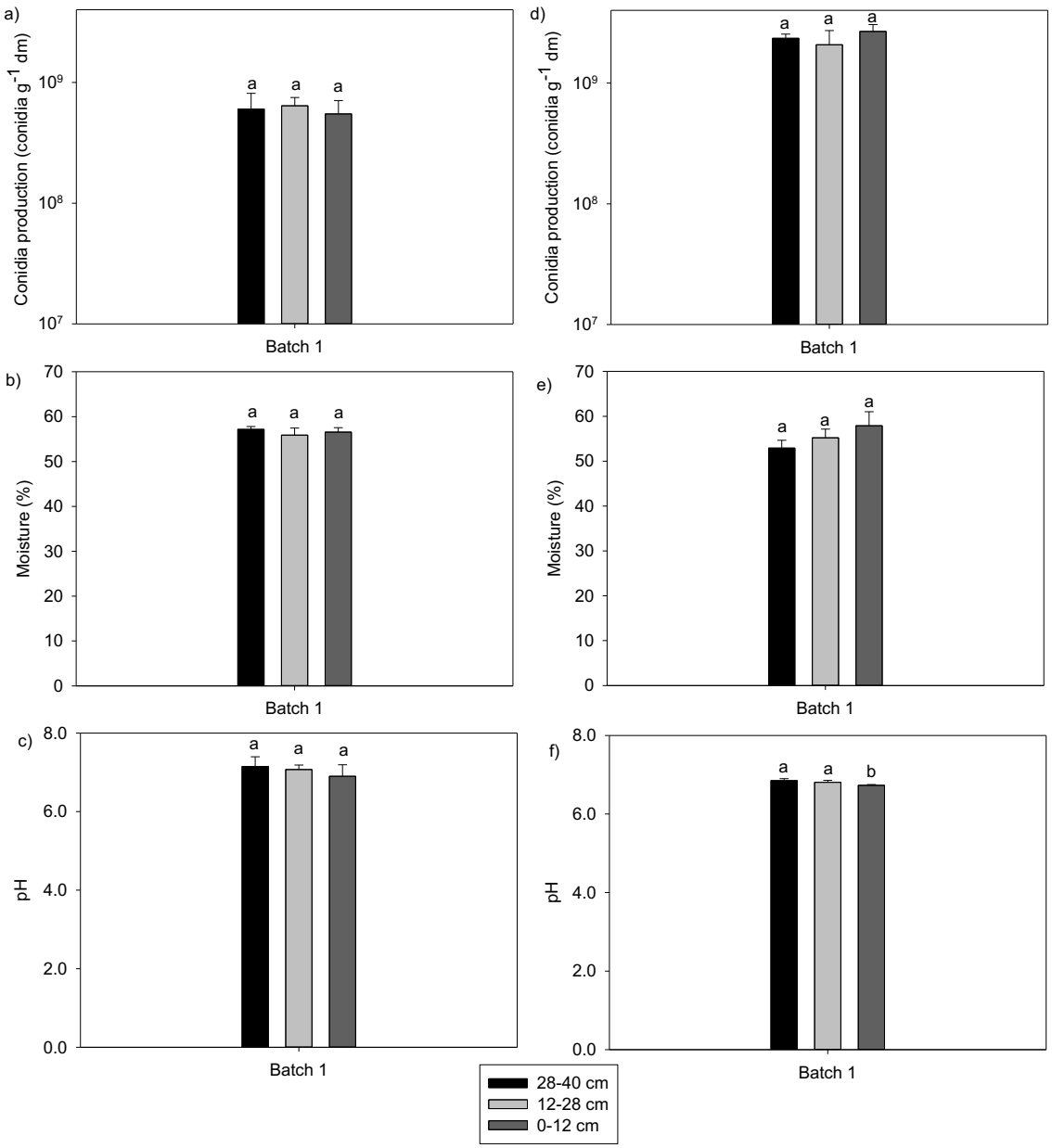



**Figure 6.22.** Mean values and standard deviations obtained for samples collected at different reactor heights at the end of all BB 22 L batches. First column corresponds to rice husk batches and second column to beer draff batches. a and d) conidia production, b and e) moisture, c and f) pH.

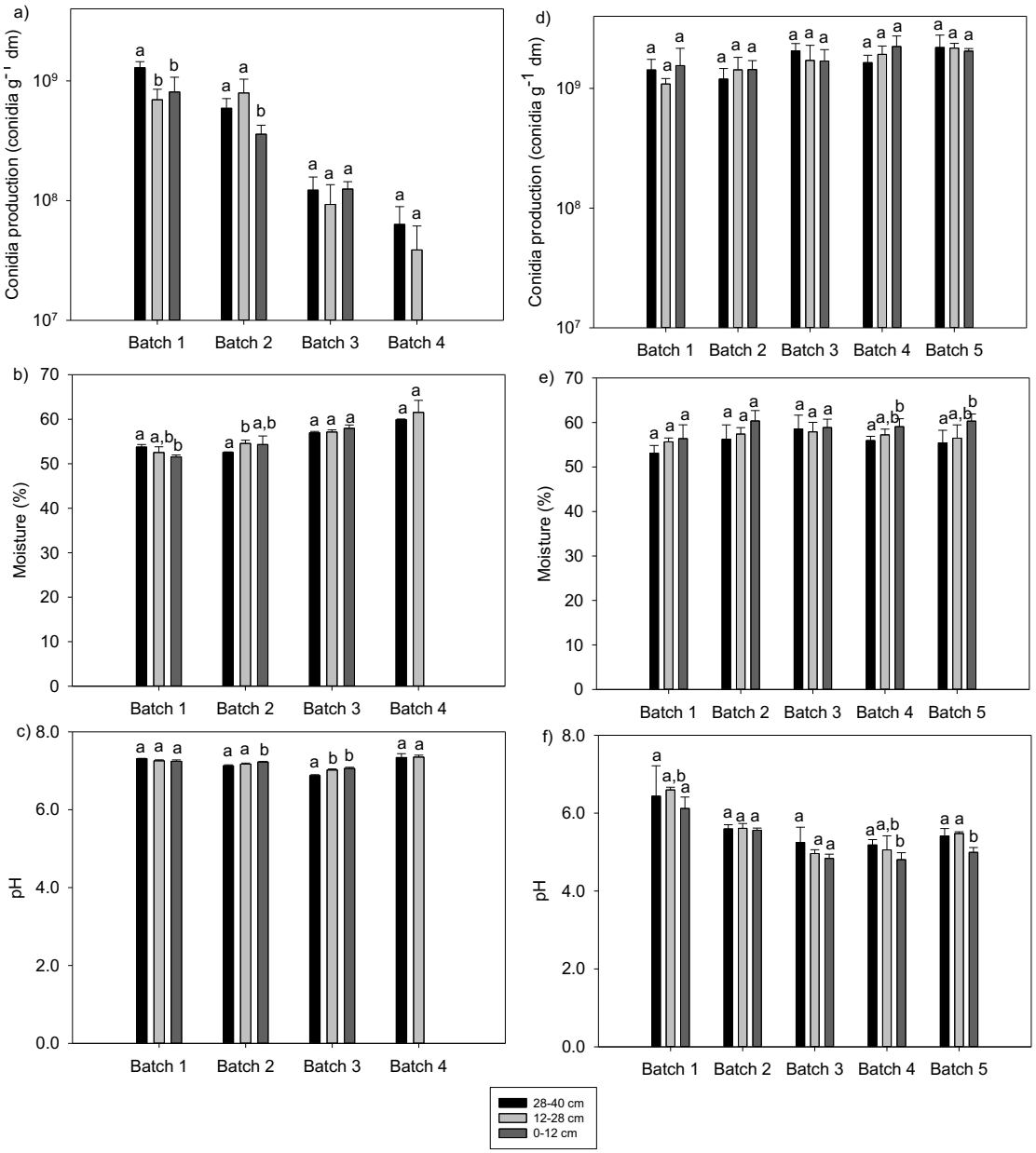



**Figure 6.23.** Mean values and standard deviations obtained in samples collected at different reactor heights at the end of all TH 22 L batches. First column corresponds to rice husk batches and second column to beer draff batches. a and d) conidia production, b and e) moisture, c and f) pH.

Temperature variation in different areas of the 22 L reactors (centre of the packed bed, close to the reactor's wall, mean values and external temperature) is shown in Figure 6.24. Despite differences between reactors and substrates, in most cases analysed temperatures were close to the optimal growth temperature range found in Chapter 4 for both BB and TH. Reactors corresponding to rice husk showed little to no difference in temperature values between different reactor areas, with minimal temperature differences

being of a maximum of 3°C, effectively achieving similar temperature on all the packed beds' volume. This behaviour is comparable to the one obtained by Barrera et al. (2019), who reported advantageous temperature axial gradients using both rice husk and polyurethane foam as substrate and inert support for *Trichoderma asperellum* conidia production. However, in beer draff batches, radial temperature differences were observed, as temperatures at the reactors' wall and the centre of the bed were different during all the fermentation process, with differences ranging from minimal 2-3°C to more than 10°C depending on fermentation time. Higher temperatures were achieved in the centre of the bed in beer draff reactors, which is consequent with the higher respiration indexes observed when using beer draff in comparison to rice husk, leading to an overall increase of the bed's temperature. Similar approach was presented by da Cunha et al. (2020) working with *Metarhizium anisopliae* and a mixture of rice and sugarcane bagasse in a similar packed bed bioreactor to the one presented in this study. However, their analysis was focused on axial temperature, finding relevant temperature differences depending on the substrate axial position, behaviour that has not been studied in this paper. As no significant differences between axial conidia productions have been found, we can assume that observed axial temperature differences did not negatively affect conidia production, although in specific moments corresponding to the maximum biological activity, differences of 10-15°C between reactor wall and centre of the packed bed were observed. Given the high influence of temperature on fungal conidia production (Papagianni, 2004), it can be assumed that observed differences did not significantly affect conidia production. Although it has not been analysed in this paper, Finkler et al. (2021) described heat transfer in a SSF packed bed reactor using data obtained at various reactor heights, demonstrating an axial uniformity of the packed bed, which might also have happened in this work using both presented substrates.

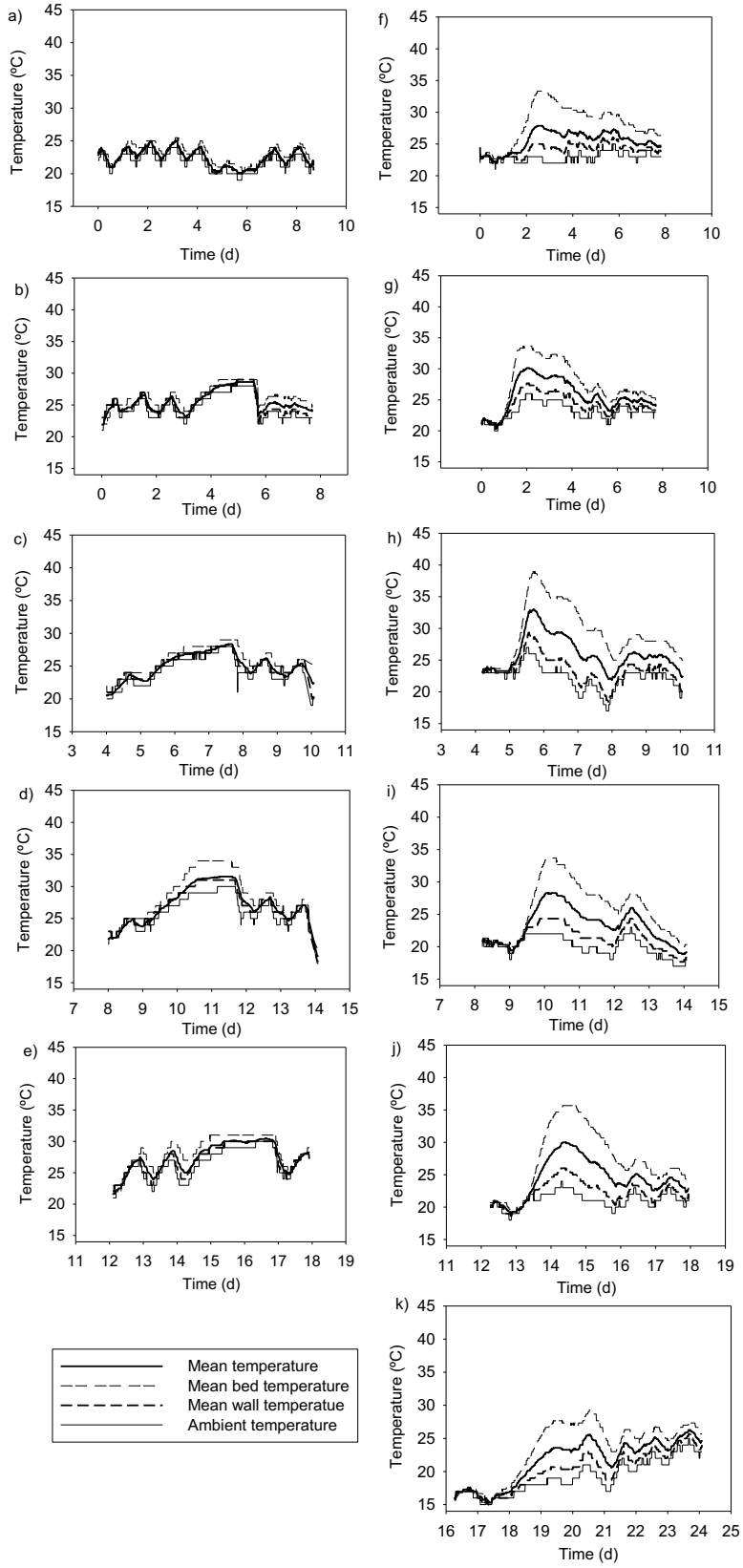



**Figure 6.24.** Temperature profiles at different areas of the 22 L reactors. Left column correspond to rice husk reactors and right column to beer draff reactors. a and f) BB reactor, b and g) TH batch 1, c and h) TH batch 2, d and i) TH batch 3, e and j) TH batch 4 and k) TH batch 5.

Figure 6.25 shows contour and mesh graphs of obtained data corresponding to last samples of all 22 L TH beer draff performed batches, presenting conidia production dependence on moisture and pH. A defined area of maximum conidia production was found, corresponding to a moisture range of 56-60% and a pH range of 5-6. Both ranges were consequent with results obtained in other works performed with similar strains (Zhang and Yang, 2015) and in Chapter 4 in this thesis.

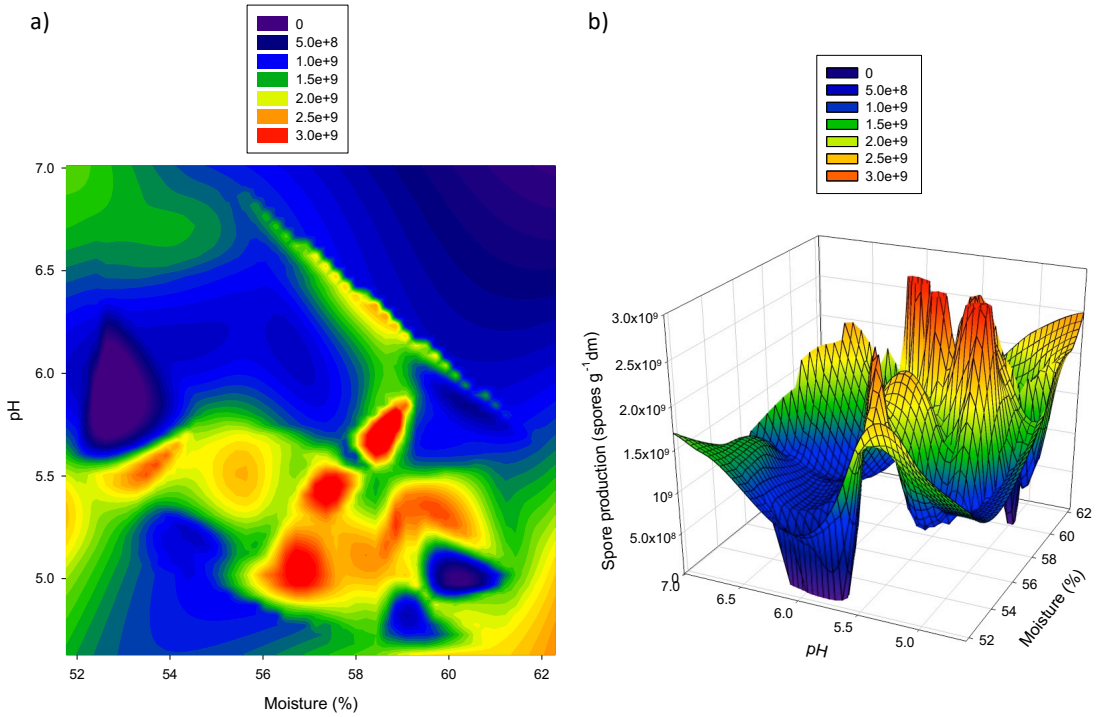



**Figure 6.25.** 3D graphics (x axis moisture, y axis pH and z axis conidia production) corresponding to results obtained in last samples of all TH 22L beer draff batches. a) Contour graph, b) 3D mesh graph.

This global analysis demonstrates that a reproducible and robust SBR process has been achieved when fermenting beer draff complemented with wood chips in 40/60% w/w proportion, as obtained results have not been significantly different for 5 consecutive batches. Aside from temperature, values of relevant parameters can be considered constant throughout the reactor bed, ensuring the robustness of the process. A minimum $AFP_R$ value of 80% is needed to correctly perform fungal fermentation at 22 L scale, as results were better in terms of consecutive batches when comparing to 70% $AFP_R$ tested at 1.5L scale (Tables 6.5 and 6.8), showing the high relevance of this parameter in the scaling process. These findings are highly relevant to fungal SSF, as most SSF processes performed using packed beds at industrial or pilot scale do not present uniformity due to

heat transfer and bed packing issues (Krishania et al., 2018), which is not only achieved in single batch in this work but also in a maximum of 5 consecutive batches by implementing a SBR strategy.

## Final remarks

A robust, reproducible, and scalable process to produce BB or TH conidia in SSF packed bed bioreactors using substrates of different biodegradability has been achieved. Process scale-up has been successful using both rice husk or beer draff and wood chips as substrates. For BB, batch strategy has been the preferable choice due to presence of unavoidable AN contamination. For TH, SBR strategy has been successful using the mixture of beer draff and wood chips, sustaining conidia production for 5 consecutive batches at values close to $2.0x10^9$ conidia $g^{-1}$dm. Prioritizing the use of the safest and sterilisable substrate, rice husk was discarded in favour of beer draff under both strategies, as beer draff AN contamination effect was lower than in rice husk when using BB or had no negative effect when working with TH, despite sterilization. Differences in performance between scales when using beer draff allowed the definition of a minimum $AFP_R$ value of 80%, determining $AFP_R$ as a key parameter in SSF process scale-up. Process robustness was demonstrated with packed bed uniformity in all 22 L reactors with both substrates and strains, despite their different biodegradability. No significant variations throughout the height of the reactor for conidia production, moisture and pH, were detected showing only minimum temperature rise when scaling beer draff. Implementing a SBR strategy with adequate $AFP_R$ values helps at overcoming major scale-up drawbacks, at least up to a scale of 22 L, being a feasible alternative to traditional batch operation when substrate contamination is bypassed.

# Chapter 7

## Tray bioreactor and global conidia production analysis

Part of this chapter was presented at 8th International Conference on Sustainable Solid Waste Management (June 2021), with the title: Sala, A., Echegaray, T., Palomas, G., Boggione, J., Tubio, G., Barrena, R., Artola, A. Insights on fungal solid-state fermentation for waste valorization: conidia and chitinase production in different reactor configurations. It has also been primarily selected for publication in Sustainable Chemistry and Pharmacy.

## 7.1. Summary/Overview

As presented in Chapter 1, fungal SSF is performed using a wide variety of reactor configurations at laboratory scale (Krishania et al., 2018; Méndez-González et al., 2018; Ramachandran et al., 2008). One of the most used, is the tray bioreactor, being preferred for most industrial processes (Nigam and Pandey, 2009., Krishania et al., 2018., Mascarin et al., 2019). When working with tray bioreactors, bed thickness is pointed as one of the most relevant parameters, as increasing thickness might result in lower conidia production (Xie et al., 2012). Important differences on bed thickness have been presented by some authors, ranging from 1 to 15 cm (Jou and Lo, 2011; Krishania et al., 2018). However, our results with PBB presented in Chapter 6 using rice husk or beer draff complemented with wood chips as a bulking agent suggest lower effect of bed thickness on conidia production if $AFP_R$ is correctly adjusted, as no relevant differences for conidia production, moisture and pH were found throughout the height (40 cm) of 22 L reactors. Preliminary tests have been conducted on this thesis on a tray bioreactor for the results to be compared to those obtained in PBB.

On the other hand, mycoparasitism, which is closely related to fungal biocontrol agents' insecticidal activity, depends on the presence of various enzymes. One of the enzymes capable of degrading lignocellulosic material (cellulases) was analysed in Chapter 4 for both strains, obtaining low results overall. When focusing on biopesticide activity, chitin content at the end of growth is considered as a good indicator of conidia formation in fungi (Desfarges et al., 1987). Chitinases partially degrade various insects, nematodes or fungi cell wall (González et al., 2010), presenting high relevance in pest control due to chitin abundance in insects, arthropods and fungi cell wall (Berini et al., 2018). Recent attention has been given to fungal biopesticides capacity to produce chitinase and other hydrolytic enzymes. In the case of *Trichoderma*, chitinase has been stated as the enzyme responsible of its biocontrol capabilities (Anand et al., 2009), whereas no studies on their role in BB conidia production have been found. Despite the relation of fungal growth and enzyme production with the fermentation conditions, there is still a lack of studies on both (Aita et al., 2019), specially related to SSF systems.

The aim of this chapter is to present an initial comparison between different reactor configurations using different substrates, analyzing data extracted from both PBBs (1.5 and 22 L) and from tray bioreactor. Data obtained from tray bioreactor tests correspond to preliminary experiments. Additionally, evolution of chitinases has been

determined in some of the tests performed in tray bioreactor. Fermentations in this Chapter were performed in collaboration with two students of Biological and Environmental Engineering UAB Master Degree (Talia Echegaray and Gonzalo Palomas) and one post-doctoral researcher from Universidad Nacional de Rosario, Argentina (Maria Julia Boggione).

## 7.2. Materials

A total of two supplies (one for rice husk and one for beer draff) were used to perform tray bioreactor tests. Substrates and wood chips (bulking agent) characterization is presented in Table 7.1.

**Table 7.1.** Characterization of rice husk and beer draff supplies and wood chips used in tray bioreactor tests.

| Parameter/supply | RH6 | BDr3 | WC2 |
|---|---|---|---|
| **MC (%)** | 10.2 ± 0.1 | 77.1 ± 0.2 | 10.4 ± 0.2 |
| **OM (%)** | 83.5 ± 1.4 | 93.3 ± 0.3 | 99.0 ± 0.8 |
| **pH** | 5.7 ± 0.3 | 6.6 ± 0.2 | 4.1 ± 0.4 |
| **Carbon (%)** | 41.0 ± 0.6 | 47.1 ± 1.1 | 48.3 ± 0.8 |
| **Hydrogen (%)** | 5.2 ± 0.1 | 6.7 ± 0.2 | 5.7 ± 0.2 |
| **Nitrogen (%)** | 0.4 ± 0.1 | 3.0 ± 0.4 | 0.4 ± 0.1 |
| **Sulphur (%)** | <0.1 | 0.2 ± 0.02 | <0.1 |
| **C/N ratio** | 111.2 ± 17.6 | 15.9 ± 1.8 | 120.8 ± 14.2 |
| **BD (kg $m^{-3}$)** | 161 ± 2 | 355 ± 5 | 109 ± 4 |
| **TSC (mg $g^{-1}$dm)** | 17.7 ± 0.3 | 118.7 ± 6.1 | 90.4 ± 7.2 |
| **$AFP_R$ (%)** | 90.3±0.5 | 63.5 ± 1.6 | 95.1 ± 0.2 |

MC: moisture content; OM: organic matter; BD: bulk density; TSC: total sugar content; $AFP_R$: air-filled porosity; RH: rice husk; BDr: beer draff; WC: wood chips

In the global analysis of results, produced conidia, mean temperature and mean sOUR values are presented. Data used for PBBs corresponds to first performed batches in SBR tests for both BB and TH 1.5 L and 22 L reactors.

## 7.3. Tests

All tray bioreactor tests were performed with the aim of comparing PBB rice husk conidia production and productivity with a different reactor configuration. In all tray bioreactor tests, analytical methods were performed by sampling two points of each tray in each sample time, except for the last sample where all the fermented material of each tray was mixed before sampling.

### 7.3.1. Rice husk tray bioreactor

Two batches were performed using set-up 1 of tray bioreactor. RH6 was used as substrate and TH as inoculum. Table 7.2. shows initial parameter values for rice husk tray bioreactor tests. No batches were performed with this configuration using BB as inoculum.

**Table 7.2.** Initial parameter values for rice husk tray bioreactor tests.

| Parameter | MC (%) | OM (%) | IC (conidia $g^{-1}dm$) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}dm$) | Ferm. time (d) |
|---|---|---|---|---|---|---|---|
| **Batch 1** | 58.5 ± 1.3 | 87.4 ± 0.4 | $6.5x10^6$ | 6.8 ± 0.3 | 1000 | 1.84 - 2.68 | 10 |
| **Batch 2** | 56.4 ± 2.2 | 84.6 ± 0.3 | $5.8x10^6$ | 6.9 ± 0.2 | 1000 | 1.75 – 2.55 | 6 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; $AFP_R$: air-filled porosity.

### 7.3.2. Beer draff tray bioreactor

Two batches were performed using set-up 2 of tray bioreactor. Using BDr3 as substrate and WC2 as bulking agent, one batch using each fungus was performed. Chitinase activity assays were performed using all samples withdrawn from both strains' tests. Table 7.3. shows initial parameter values for beer draff tray bioreactor tests. To avoid water condensate generation observed in previous tests, air was not moistened before entering the reactor in TH batch.

**Table 7.3.** Initial parameter values for beer draff tray bioreactor tests.

| Parameter/ Batch | MC (%) | OM (%) | IC (conidia $g^{-1}dm$) | pH | AF (mL $min^{-1}$) | sAF (mL $min^{-1}$ $g^{-1}dm$) | Mixture (BDr/WC) (%/%) | $AFP_R$ (%) |
|---|---|---|---|---|---|---|---|---|
| **BB** | 63.4 ± 0.7 | 97.2 ± 0.3 | $4.6x10^6$ | 4.9 ± 0.2 | 500 | 0.76 – 0.91 | 70/30 | 70 |
| **TH** | 66.1 ± 1.1 | 98.0 ± 0.6 | $6.1x10^6$ | 5.0 ± 0.2 | 1000 | 1.09 – 1.31 | 70/30 | 71 |

MC: moisture content; OM: organic matter; IC: inoculum concentration; AF: airflow; BDr: beer draff; WC: wood chips; $AFP_R$: air-filled porosity; BB: *Beauveria bassiana*; TH: *Trichoderma harzianum*.

## 7.4. Results and discussion

### 7.4.1. Rice husk TH tray bioreactor

Rice husk weight in each tray was 450 g, equaling the mass of this substrate treated in 1.5 L PBB.

Figure 7.1. presents the results of rice husk TH tray bioreactor test 1 whereas Figure 7.2 presents the results of fermentation 2. Table 7.4 presents ranges for various parameters for both tests. Main difference between both tests was fermentation time, being of 10 days in test 1 and of 6 days in test 2 (Table 7.2). Figure 7.3 presents tray appearance of one of the fermented trays in test 1.

Figures 7.1 and 7.2 show similar performance between duplicates. As maximum conidia production in terms of productivity was found at approx. 6 days in test 1, test 2 was run for 6 days also confirming this maximum. This value, corresponds to optimal conidia production time found in Chapter 4. Similar conidia profiles were obtained between trays in both tests, with values ranging from $3.7x10^8$ to $8.3x10^8$ conidia $g^{-1}dm$. Maximum conidia production was obtained in different trays in each test, meaning tray position did not adversely affect conidia production. However, conidia concentrations were lower in all cases than those obtained in PBBs, which could be due to the use of non-optimized air distribution, causing possible $O_2$ deficiency in some parts of the reactor. However, this hypothesis could not be confirmed, as no respiration profile was obtained in both fermentations. This behaviour might be related to the direction of the air sprinklers, which were faced up in this fermentation according to set-up 1 shown in Chapter 3. Consequently, the tray bioreactor design was changed from Figure 3.16 c) to Figure 3.16 d) in the rest of the presented tray bioreactor tests.

Mean temperature profile showed an increase during the first 3 days in test 1, while similar increase was shown in day 3 in test 2, stabilising in values close to the optimal of 25ºC for the rest of both fermentations. The low biodegradability of rice husk (resulting in very low respiration values as presented in Chapters 4 and 6) difficulted monitoring respiration profiles. Much of the incubator's volume was not filled with substrate, leaving a huge dead volume. However, specific airflow in this test was the highest in the thesis (1.68-2.55 mL $min^{-1}$ $g^{-1}$dm), much higher than Chapter 6 values using the same substrate (maximum of 0.6 mL $min^{-1}$ $g^{-1}$dm). Thus, air leaks, coupled with rice husk's low respiration values, might be the reasons behind not obtaining respiration profiles in both tests.

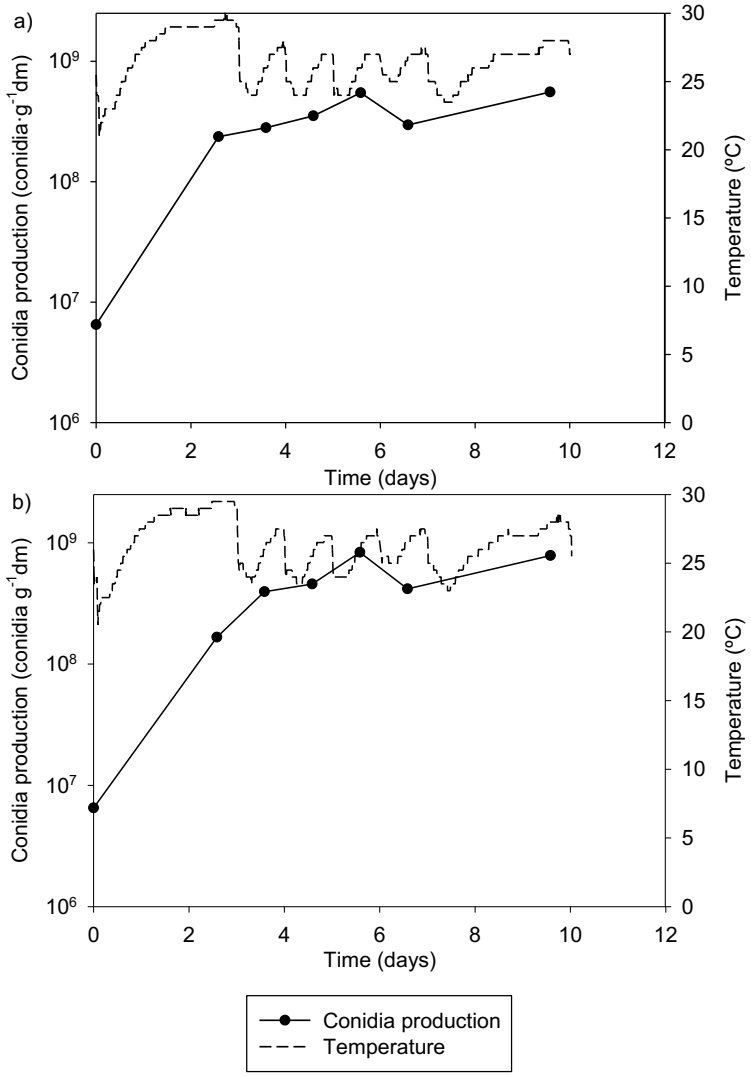



**Figure 7.1.** Process parameters evolution in TH tray bioreactor test 1 using rice husk. a) Tray 1. b) Tray 2. Temperature profile in both graphs corresponds to whole reactor profile.

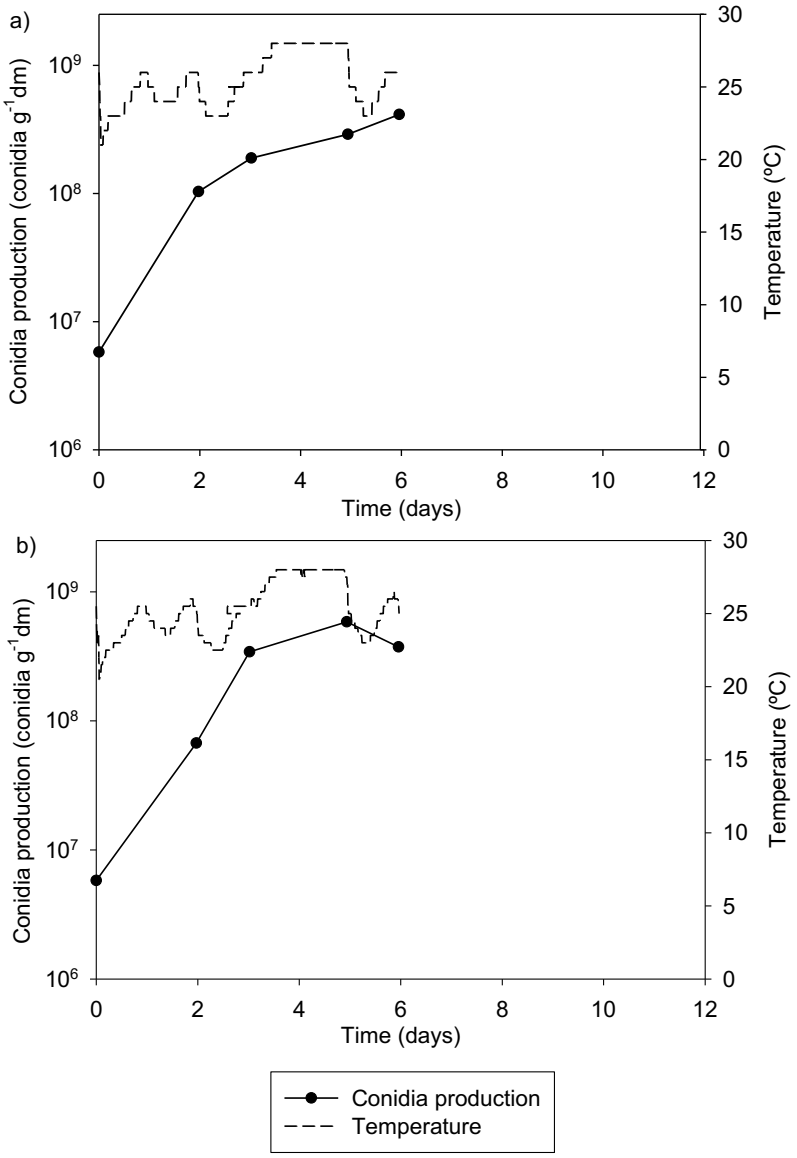



**Figure 7.2.** Process parameters evolution in TH tray bioreactor test 2 using rice husk. a) Tray 1. b) Tray 2. Temperature profile in both graphs corresponds to whole reactor profile.

**Figure 7.3.** Example of tray appearance in TH rice husk tray bioreactor test.

As shown in Table 7.4, similar values between trays were found for moisture, pH and total sugar content. Although only initial and final values were analysed for both moisture and pH, obtained values suggest similar behaviour to observed profiles in Chapters 4 and 6 using the same substrate. Moisture initial and final values were similar in most trays, suggesting moisture maintenance thanks to the use of saturated air. Total sugar content values were also similar (test 1), suggesting possible similarities in sugar consumption profiles.

**Table 7.4.** Moisture content, pH and total sugar content initial and final values in TH tray bioreactor (test 1 and 2). Maximum productivity and conidia quotient are also shown. Different superscripts indicate statistical difference.

| Parameter/ Batch | Tray 1 Test 1 | Tray 2 Test 1 | Tray 1 Test 2 | Tray 2 Test 2 | Mean values |
|---|---|---|---|---|---|
| MC (%) | $58.5 \pm 0.7^{(a)}$ – $56.1 \pm 0.4^{(b)}$ | $58.5 \pm 0.7^{(a)}$ – $56.3 \pm 0.3^{(b)}$ | $56.4 \pm 1.8^{(a,b,c)}$ – $57.4 \pm 0.3^{(c)}$ | $56.4 \pm 1.8^{(a,b,c)}$ – $56.8 \pm 0.3^{(b)}$ | $57.5 \pm 1.3$ – $56.7 \pm 0.6$ |
| pH | $6.8 \pm 0.2^{(a)}$ – $7.6 \pm 0.1^{(b)}$ | $6.8 \pm 0.2^{(a)}$ – $7.6 \pm 0.2^{(b)}$ | $6.9 \pm 0.1^{(a)}$ – $7.5 \pm 0.2^{(b)}$ | $6.9 \pm 0.1^{(a)}$ – $7.5 \pm 0.2^{(b)}$ | $6.9 \pm 0.1$ – $7.6 \pm 0.2$ |
| TSC (mg $g^{-1}$dm) | $13.3 \pm 1.4^{(a)}$ – $3.9 \pm 0.4^{(b)}$ | $13.3 \pm 1.4^{(a)}$ – $3.5 \pm 0.3^{(b)}$ | (-) | (-) | $13.3 \pm 1.4$– $3.7 \pm 0.2$ |
| CPr (conidia $g^{-1}$dm $d^{-1}$) | $9.8x10^7 \pm 1.2 x10^{7\,(a)}$ | $8.2x10^7 \pm 9.4x10^{6\,(a,b)}$ | $7.0 x10^7 \pm 8.7 x10^{6\,(b)}$ | $1.5x10^8 \pm 2.3x10^{7\,(c)}$ | $1.0x10^8 \pm 3.5x10^7$ |
| CQ | 83 | 129 | 62 | 88 | 90.5 ± 28 |

MC: moisture content; TSC: total sugar content; CPr: conidia productivity; CQ: conidia quotient

Overall, rice husk tray bioreactor behaviour (with the only exception of total sugar consumption) was similar to that observed when working with 1.5 L PBBs (total quantity of loaded material in tray bioreactor was the same as in 1.5 L PBB triplicate). Little differences were found between trays and fermentations, suggesting no effect of tray position in conidia production. Results among reactor configurations (PPB and tray) using rice husk as substrate were comparable when inoculating with TH.

### 7.4.2. Beer draff BB tray bioreactor

Results corresponding to beer draff BB tray bioreactor test are presented in Figure 7.4 and Table 7.5. Figure 7.4 shows conidia and chitinase production profile for each tray,

while both sOUR and temperature are shown as mean values. Tray reactor set-up 2 holding 3 trays was used in this test. Appearance of one tray is shown in Figure 7.5.

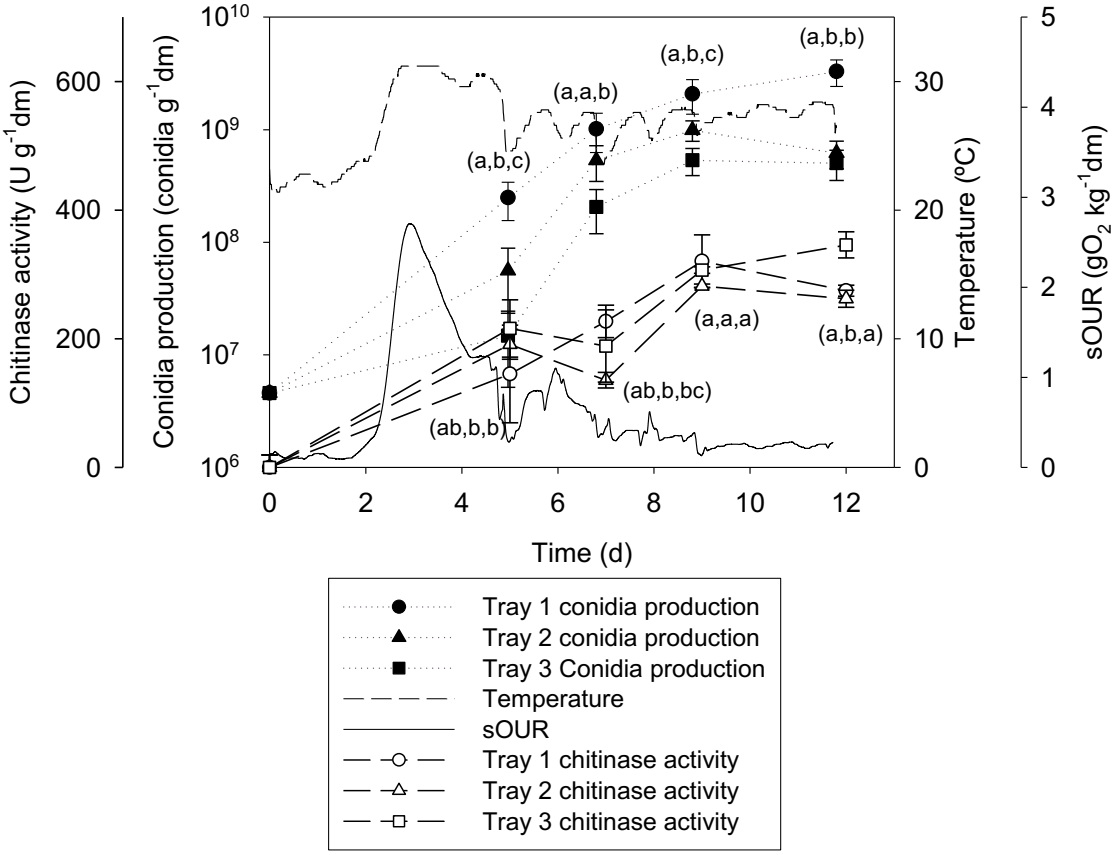



**Figure 7.4.** Process parameters evolution in BB beer draff tray bioreactor. Produced conidia and chitinase concentration are shown for each tray. Statistical difference between trays is shown in format (tray 1, tray 2, tray3).

**Figure 7.5.** Example of tray appearance in BB beer draff tray bioreactor test.

Significant conidia production differences in at least two out of three trays were observed in all samplings. Although maximum conidia production was reached at 11.8 d in tray 1, trays 2 and 3 reached its maximum at day 8.8. As such, assuming 8.8 days as

maximum conidia production time (at least for two out of three trays), conidia production for tray 1 was of $2.1x10^9$ conidia $g^{-1}$dm, of $9.9x10^8$ conidia $g^{-1}$dm for tray 2 and of $5.4x10^8$ conidia $g^{-1}$dm for tray 3. Mean value of the three trays was $1.2x10^9 \pm 7.9x10^8$ conidia $g^{-1}$dm. This behaviour cannot be explained by lack of $O_2$ in trays 2 and 3, as $O_2$ percentages for the whole reactor only dropped to values close to 15%, demonstrating sufficient $O_2$ availability for all trays in the whole fermentation. However, differences between trays could be explained by air distribution through the reactor, as preferred paths might have been originated, difficulting air distribution for trays located further from the sprinklers. It is also possible that changes to air dispersion caused by the use of a different tray bioreactor set-up (facing the sprinklers down) might have influenced conidia production. However, as same substrate and strain were not tested using both set-ups, this statement remains as a hypothesis. Achieved conidia production was similar to the one obtained by Xie et al. (2013) using rice as substrate, confirming the potential of beer draff.

Similar chitinase activity profiles were achieved in all trays, with maximum chitinase production time being of around 9 days. Chitinase activities were not significantly different between trays in most of the analysed samples. Highest values in all trays were near 300 U $g^{-1}$dm. Maximum chitinase activity was achieved at the same time of maximum conidia productivity. In most of the presented BB fermentations in this thesis, this optimum has been of 7.5-8 days. With these results, this optimal could still be possible in this test. However, this statement cannot be assumed, as there is no analysis between 6.8 and 8.8 days, meaning maximum chitinase production could be achieved after maximum conidiation, as suggested by Desfarges et al (1987). Contrary to observed behaviour in conidia production, airflow role in chitinase production seemed independent due to not presenting significant differences between trays. As stated in section 7.1, no studies on chitinase role in BB conidia production have been found previous to this thesis.

In terms of biodegradability, both maximum temperature and maximum sOUR were achieved at the same time (aprox. day 3), with sOUR immediately decreasing but observing temperature maintenance until day 4.5-5. Maximum sOUR reached 2.7 $gO_2$ $kg^{-1}$dm, being similar to values obtained in Chapter 6 using the same substrate mixture. Lag phase of almost 2 days was also present, indicating need of adaptation to the substrate prior to the start of fungal growth. As shown in Figure 7.6, temperature profiles showed same tendency in all trays. Maximum temperature did not surpass 32ºC in tray 3, being only 3ºC superior to tray 1 at the moment of maximum biological activity. However, temperature differences might have reduced conidia production in tray 3 due to

temperature relevance in fungal conidia production. As demonstrated in previous Chapters, BB strain used in this thesis is susceptible to temperature changes, even if they are only of 3ºC. Consequently, this difference might have negatively affected conidia production in trays located further from the sprinklers due to having lower heat dissipation.

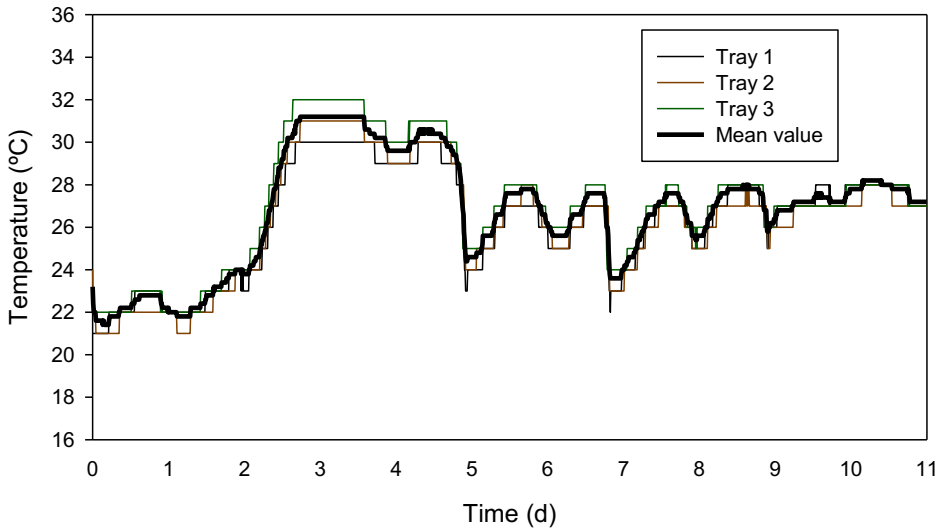



**Figure 7.6.** Temperature profiles obtained on each tray in beer draff BB tray bioreactor. Mean value from all trays is shown in bold.

As shown in Table 7.5, moisture content, pH and total sugar content values were very similar between all trays, only showing significant difference in tray 3 final moisture values, corresponding to the tray located further from the sprinklers. However, moisture values in tray 3 were closer to optimal for BB conidia production, according to our results in Chapter 4. Both moisture and pH ranges were kept within optimums found in previous chapters. Consequently, moisture and pH did not negatively affect conidia production, leaving temperature and air distribution as the causing behind observed differences.

**Table 7.5.** Moisture content, pH and total sugar content range in BB beer draff tray batch. Maximum productivity and conidia quotient are also shown. Different superscripts indicate statistical difference.

| Parameter/ Batch | Tray 1 | Tray 2 | Tray 3 | Mean values |
|---|---|---|---|---|
| **MC range (%)** | $63.4 \pm 0.7^{(a)}$ – $58.2 \pm 0.7^{(b)}$ | $63.4 \pm 0.7^{(a)}$ – $59.3 \pm 0.4^{(b)}$ | $63.4 \pm 0.7^{(a)}$ – $62.6 \pm 1.2^{(a)}$ | $63.4 \pm 0.7$ – $60.0 \pm 2.3$ |
| **pH range** | $4.9 \pm 0.2^{(a)}$ – $7.2 \pm 0.3^{(b)}$ | $4.9 \pm 0.2^{(a)}$ – $7.4 \pm 0.2^{(b)}$ | $4.9 \pm 0.2^{(a)}$ – $7.2 \pm 0.1^{(b)}$ | $4.9 \pm 0.2$ – $7.3 \pm 0.1$ |
| **TSC range (mg $g^{-1}$dm)** | $99.4 \pm 6.7^{(a)}$ – $17.3 \pm 1.2^{(b)}$ | $99.4 \pm 6.7^{(a)}$ – $16.2 \pm 2.1^{(b)}$ | $99.4 \pm 6.7^{(a)}$ – $19.7 \pm 0.9^{(c)}$ | $99.4 \pm 6.7$ – $17.7 \pm 1.8$ |
| **CPr (conidia $g^{-1}$dm $d^{1}$)** | $2.4x10^{8} \pm 5.2x10^{7(a)}$ | $1.1x10^{8} \pm 4.6x10^{7(b)}$ | $6.1x10^{7} \pm 1.7x10^{7(c)}$ | $1.3x10^{8} \pm 9.0x10^{7}$ |
| **CQ** | 453 | 216 | 117 | $262 \pm 173$ |

MC: moisture content; TSC: total sugar content; CPr: conidia productivity; CQ: conidia quotient

### 7.4.3. Beer draff TH tray bioreactor

Results corresponding to beer draff TH tray bioreactor test are presented in Figure 7.7 and Table 7.6. Figure 7.7 shows conidia and chitinase production profile for each tray, while both sOUR and temperature are shown as mean value. Tray reactor set-up 2 was used in this test. Appearance of one tray is shown in Figure 7.8.

Opposing to BB results shown in section 7.4.2, no significant differences were observed between most of conidia productions in the different samples. Maximum conidia production was obtained in day 6 and stabilized afterwards, being the same optimum conidia productivity time found in TH PBBs tests presented in previous chapters and for RH in tray bioreactor presented above. Maximum conidia production was of $3.0x10^{9}$ conidia $g^{-1}$dm for tray 1, $2.0x10^{9}$ conidia $g^{-1}$dm for tray 2 and $2.5x10^{9}$ conidia $g^{-1}$dm for tray 3 (mean value, $2.5x10^{9} \pm 4.9x10^{8}$ conidia $g^{-1}$dm). Although maximum conidia production was still achieved in tray 1, it was not significantly different in comparison to the rest, suggesting no relevant effect of distance from air inlet when using TH, same behaviour previously found when using rice husk as substrate. As it happened in previous Chapters in this work, used TH strain demonstrated higher versatility in comparison to the used BB strain.

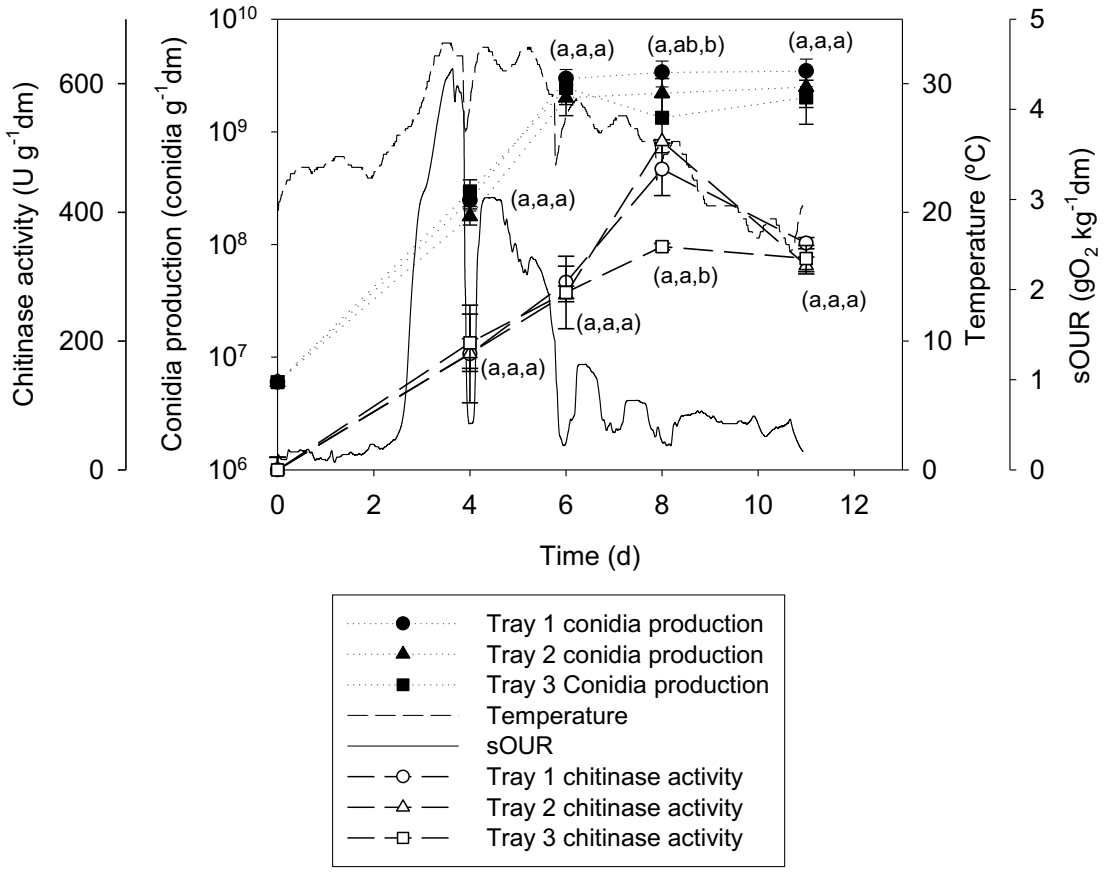



**Figure 7.7.** Process parameters evolution in TH beer draff tray bioreactor. Conidia and chitinase concentrations are shown for each tray. Statistical difference between trays is shown in format (tray 1, tray 2, tray3).

**Figure 7.8.** Example of tray appearance in TH beer draff tray bioreactor test.

Similar chitinase activity profiles were achieved in all trays, with maximum chitinase production time being around 8 days. Chitinase activities were not significantly different between trays in most of the analysed samples, with the only exception of maximum chitinase production time. Highest values were achieved in trays 1 and 2 (465-

510 U $g^{-1}$dm), being significantly different from tray 3 chitinase activity of 350 U $g^{-1}$dm. Similar to BB test, maximum chitinase activity was achieved after maximum conidia production (2 days). TH chitinase profiles were similar to the ones obtained by Sandhya et al. (2004) using SmF and mycelia as inoculum, opposed to conidia used in this work. At maximum production time, chitinase production was different between trays, as the lowest activity was achieved in the tray located further from the air sprinklers, being significant in comparison to the rest. However, this difference was not observed in any other sampling. Although positive airflow influence when producing chitinases had been previously observed by other authors using various *Trichoderma* strains (De la Cruz Quiroz et al., 2017), it cannot be assumed that distance from airflow has negatively affected chitinase production when observing this difference only at one sampling time. More experiments should be performed before reaching a final conclusion on its importance. These studies should aim at maximizing both chitinase and conidia concentrations.

In terms of biodegradability, both temperature and sOUR profiles were similar to the ones shown in BB beer draff tray bioreactor test, including the lag phase duration. This result suggests similar adaptation times for both fungi despite TH achieving maximum conidia production before BB, as in all previous tests. Maximum sOUR values were achieved at the same time but showing higher values (4.2 $gO_2$ $kg^{-1}$dm), being 1.5 times superior to BB maximum with the same substrate. These values were comparable to observed values in Chapter 6 using the same substrate mixture. Temperature profiles of all trays are presented in Figure 7.9. Differences between trays in terms of temperature were found at maximum activity time, being of a maximum of 5ºC between tray 1 and tray 3 (30 to 35ºC). However, when temperatures were not at its peak, differences between trays were of a maximum of 2ºC. Despite temperature relevance in conidia production, these differences did not negatively affect conidia production in tray 3, opening the possibility of fermenting more quantity of material per tray. This behaviour is noteworthy to mention, as higher temperature differences between trays (or even in the same tray) would be expected in case of increasing bed thickness (Krishania et al., 2018; Xie et al., 2013., Jou and Lo, 2011).

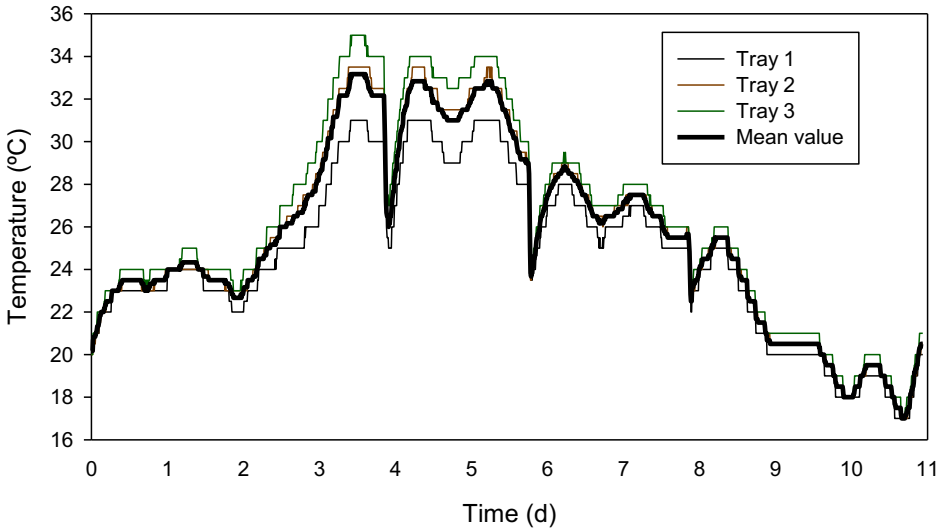



**Figure 7.9.** Temperature profiles obtained on each tray in beer draff TH tray bioreactor. Mean value from all trays is shown in bold.

As shown in Table 7.6, moisture content, pH and total sugar content were very similar between all trays, only showing significant difference between trays in tray 1 final moisture values. pH range was kept within optimums found in previous chapters, while total sugar content values are similar to the ones observed in previous tests. Moisture differences between tray 1 and trays 2 and 3 were mainly caused by the use of non-moistened air in this test to avoid the formation of water condensates. Consequently, relative humidity inside the tray bioreactor chamber could not be controlled, causing substrate moisture reduction (Krishania et al., 2018). Additionally, these differences might have been amplified by tray 1 being the closest to the air sprinklers, resulting in higher moisture reduction in this tray in comparison to the rest. However, moisture reduction did not diminish conidia or chitinase production in tray 1, confirming the versatility of the used TH strain, maintaining similar production even when moisture could not be kept at optimal values.

**Table 7.6.** Moisture content, pH and total sugar content initial and final values in TH beer draff tray batch. Maximum productivity and conidia quotient are also shown. Different superscripts indicate statistical difference.

| Parameter/ Batch | Tray 1 | Tray 2 | Tray 3 | Mean values |
|---|---|---|---|---|
| **MC (%)** | $66.1 \pm 1.1^{(a)}$ – $48.3 \pm 0.6^{(b)}$ | $66.1 \pm 1.1^{(a)}$ – $56.1 \pm 1.4^{(c)}$ | $66.1 \pm 1.1^{(a)}$ – $58.0 \pm 0.9^{(c)}$ | $66.1 \pm 1.1$ – $54.1 \pm 5.1$ |
| **pH** | $4.9 \pm 0.2^{(a)}$ – $7.5 \pm 0.3^{(b)}$ | $4.9 \pm 0.2^{(a)}$ – $7.6 \pm 0.2^{(b)}$ | $4.9 \pm 0.2^{(a)}$ – $7.8 \pm 0.2^{(b)}$ | $4.9 \pm 0.2$ – $7.6 \pm 0.2$ |
| **TSC (mg $g^{-1}$dm)** | $103.5 \pm 3.8^{(a)}$ – $17.6 \pm 0.8^{(b)}$ | $103.5 \pm 3.8^{(a)}$ – $18.4 \pm 0.5^{(b)}$ | $103.5 \pm 3.8^{(a)}$ – $18.0 \pm 0.6^{(b)}$ | $103.5 \pm 3.8$ – $18.0 \pm 0.4$ |
| **CPr (conidia $g^{-1}$dm $d^{1}$)** | $5.0x10^{8} \pm 9.3x10^{7(a)}$ | $3.4x10^{8} \pm 8.1x10^{7(a)}$ | $4.1x10^{8} \pm 7.5x10^{7(a)}$ | $4.1x10^{8} \pm 8.1x10^{7}$ |
| **CQ** | 497 | 335 | 410 | $414 \pm 81$ |

MC: moisture content; TSC: total sugar content; CPr: conidia productivity; CQ: conidia quotient

## 7.5. Global analysis

This section presents a comparison between some of the tests using PBBs in Chapter 6 and tray bioreactor tests in this chapter for both BB or TH. All conidia production values in this section correspond to mean values at maximum conidia production time.

Table 7.7 results allow a comparison based on parameters other than conidia production (conidia $g^{-1}$dm). While in terms of productivity (conidia $g^{-1}$dm $d^{-1}$) results follow a similar pattern when comparing with conidia production, different patterns are observed with total produced conidia and total produced conidia $L^{-1}$.

**Table 7.7.** Conidia production, productivities and conidia quotients of all fermentations.

| Test/parameter | Produced conidia (conidia $g^{-1}dm$) | Time (d) | Grams dry matter (g dm) | Total volume (L) | sAF (mL $min^{-1}$ $g^{-1}dm$) | Productivity (conidia $g^{-1}dm\ d^{-1}$) | Total produced conidia | Total produced conidia $L^{-1}$ | CQ |
|---|---|---|---|---|---|---|---|---|---|
| **Tray bioreactor RH TH** | $7.0x10^8$ | 5.8 | 387 | 43.5 | 1.75 – 2.68 | $1.2x10^8$ | $2.7x10^{11}$ | $6.2x10^9$ | 107 |
| **Tray bioreactor BDr BB** | $1.2x10^9$ | 8.8 | 540 | 43.5 | 0.76 – 0.91 | $1.4x10^8$ | $6.5x10^{11}$ | $1.5x10^{10}$ | 182 |
| **Tray biorreactor BDr TH** | $2.5x10^9$ | 5.8 | 765 | 43.5 | 1.09 – 1.31 | $4.3x10^8$ | $1.9x10^{12}$ | $4.4x10^{10}$ | 376 |
| **Packed bed 1.5 L RH BB** | $3.8x10^8$ | 7.8 | 105 | 1.5 | 0.18-0.33 | $4.5x10^7$ | $4.0x10^{10}$ | $2.7x10^{10}$ | 58 |
| **Packed bed 1.5 L RH TH** | $2.0x10^9$ | 5.8 | 105 | 1.5 | 0.18-0.33 | $3.4x10^8$ | $2.1x10^{11}$ | $1.4x10^{11}$ | 300 |
| **Packed bed 1.5 L BDr BB** | $1.5x10^9$ | 7.8 | 105 | 1.5 | 0.71–0.96 | $1.9x10^8$ | $1.5x10^{11}$ | $1.0x10^{11}$ | 222 |
| **Packed bed 1.5 L BDr TH** | $2.1x10^9$ | 5.8 | 105 | 1.5 | 0.71–0.96 | $3.6x10^8$ | $2.2x10^{11}$ | $1.4x10^{11}$ | 312 |
| **Packed bed 22 L RH BB** | $6.0x10^8$ | 7.8 | 1050 | 22 | 0.28-0.42 | $7.7x10^7$ | $6.3x10^{11}$ | $2.9x10^{10}$ | 91 |
| **Packed bed 22 L RH TH** | $9.1x10^8$ | 5.8 | 1050 | 22 | 0.28-0.42 | $1.6x10^8$ | $9.6x10^{11}$ | $4.4x10^{10}$ | 138 |
| **Packed bed 22 L BDr BB** | $2.5x10^9$ | 7.8 | 1400 | 22 | 0.53-0.87 | $3.2x10^8$ | $3.5x10^{12}$ | $1.6x10^{11}$ | 378 |
| **Packed bed 22 L BDr TH** | $1.7x10^9$ | 5.8 | 1400 | 22 | 0.53-0.87 | $2.9x10^8$ | $2.4x10^{12}$ | $1.1x10^{11}$ | 254 |

sAF: specific airflow; CQ: conidia quotient; RH: rice husk; BDr: beer draff; BB: *Beauveria bassiana*; TH: *Trichoderma harzianum*.

When total conidia production is not corrected with total volume, 1.5 L reactors show lowest obtained productions, as expected from the lowest volume. When comparing tray results with same substrate in 22 L PB configuration, total conidia production obtained are, most of the times, in the same order of magnitude, taking into account differences in fermented substrate quantity. These results suggest similar conidia productions can be reached with both configurations. When correcting total conidia production using total volume, both 1.5 and 22 L PBB show higher values in comparison to tray in the majority of the reactors. This behaviour is explained by differences in working volume between reactors: while almost all the PB volume in both configurations was used as working volume, only 8 (rice husk) or 13.5 (beer draff) out of 43.5 L were used in tray configurations. As such, better spatial usage of tray configuration might improve conidia production, with possibility of reaching higher values. It must also be considered that, when working with beer draff, the substrate/bulking agent mixture used in tray and 22 L was different (70/30 in tray vs 40/60 in 22 L packed bed w/w beer draff/wood chips). Results could vary if using same proportions, which might be needed in case of increasing bed height, which was kept constant at 4 cm in all tray bioreactor tests. According to some authors, this value could be higher without compromising airflow through the bed (Jou and Lo, 2011; Krishania et al., 2018). Increasing bed height up to the same working volume used in the 22 L packed-bed (corresponding to 8 cm bed height per tray) configuration should lead to a better comparison.

Comparing specific airflow rates, values were proportional between PBB scales. In tray fermentations, much higher values were provided for rice husk fermentations, despite its higher $AFP_R$ in comparison to beer draff. However, conidia production was much higher in TH beer draff tray fermentation rather than in rice husk. This results confirm the relevance of biodegradability as a key parameter in TH fermentations, as found in PCA analysis results in Chapter 5.

Tables 7.8 and 7.9 present a comparison of conidia production and biodegradability, including mean values for conidia production and temperature (with the correspondent statistical analyses) and maximum sOUR for the fermentations.

**Table 7.8.** Conidia production, mean temperature and maximum sOUR obtained in all BB fermentations. Statistical analyses are shown for conidia production and mean temperature.

| Test/parameter | Conidia production (conidia $g^{-1}dm$) | Mean temperature (ºC) | Max sOUR ($gO_2\ kg^{-1}dm$) |
|---|---|---|---|
| **Packed bed 1.5 L RH BB** | $3.8x10^8 \pm 1.7x10^{7(a)}$ | $26.4 \pm 0.9^{(a)}$ | 0.2 |
| **Packed bed 22 L RH BB** | $6.0x10^8 \pm 5.4x10^{7(b)}$ | $22.5 \pm 1.3^{(b)}$ | 0.7 |
| **Tray bioreactor BDr BB** | $1.2x10^9 \pm 8.0x10^{8(c)}$ | $26.9 \pm 2.6^{(a)}$ | 2.7 |
| **Packed bed 1.5 L BDr BB** | $1.5x10^9 \pm 3.8x10^{8(c)}$ | $26.0 \pm 1.7^{(a)}$ | 3.6 |
| **Packed bed 22 L BDr BB** | $2.5x10^9 \pm 6.5x10^{8(c,d)}$ | $25.5 \pm 1.6^{(a,b)}$ | 3.5 |

**Table 7.9.** Conidia production, mean temperature and maximum sOUR obtained in all TH fermentations. Statistical analyses are shown for conidia production and mean temperature.

| Test/parameter | Conidia production (conidia $g^{-1}dm$) | Mean temperature (ºC) | Max sOUR ($gO_2\ kg^{-1}dm$) |
|---|---|---|---|
| **Tray bioreactor RH TH** | $7.0x10^8 \pm 2.1x10^{8(a)}$ | $25.8 \pm 1.9^{(a,b,c)}$ | (-) |
| **Packed bed 1.5 L RH TH** | $2.0x10^9 \pm 3.0x10^{8(b)}$ | $23.0 \pm 1.9^{(a,b)}$ | 0.7 |
| **Packed bed 22 L RH TH** | $9.1x10^8 \pm 3.1x10^{8(a)}$ | $25.8 \pm 1.8^{(a,b,c)}$ | 0.7 |
| **Tray bioreactor BDr TH** | $2.5x10^9 \pm 4.8x10^{8(b)}$ | $25.6 \pm 4.4^{(a,b,c)}$ | 4.5 |
| **Packed bed 1.5 L BDr TH** | $2.1x10^9 \pm 3.7x10^{8(b)}$ | $29.1 \pm 2.6^{(a,c)}$ | 3.2 |
| **Packed bed 22 L BDr TH** | $2.1x10^9 \pm 3.6x10^{8(b)}$ | $25.7 \pm 2.6^{(a,b,c)}$ | 3.3 |

Comparing between substrates, conidia productions on rice husk were always lower compared to those on beer draff using the same configuration and strain. This behaviour was coupled with much lower biodegradability presented by rice husk in comparison to beer draff. Rice husk respiration values never surpassed 0.75 $gO_2\ kg^{-1}dm$ at both tested scales, while beer draff varied between 2.70 to a maximum of 4.45 $gO_2\ kg^{-1}dm$ in tray bioreactor. Comparing initial values from sections 6.3 and 7.3, initial parameters at the start of the fermentation were similar between substrates (with the only exception of $AFP_R$ in beer draff fermentations, determined as key parameter in beer draff process scale up in Chapter 6). These results suggest conidia production is highly dependant on substrate biodegradability.

Comparing between strains, BB and TH present different behaviours. Although significant differences between reactor configurations were observed, they were not

always between the same reactors. Maximum mean conidia production achieved using BB and beer draff was obtained in 22 L fermentation, while both tray and 1.5 L fermentations obtained more similar values. This result is remarkable, as most of BB aerial conidia production is not performed using packed-bed bioreactors as fermenters but by superficial production, which ranges from polypropylene bags and tray bioreactors to environmentally-prepared chambers for fungal growth and sporulation (Jaronski and Mascarin, 2017). The use of different substrate/bulking agent ratios between 22 L beer draff bioreactors and the rest of the tested conformations (which results in different $AFP_R$ values, as presented in Chapter 6) could possibly have affected conidia production. Use of $AFP_R$ values around 80% (Chapter 6) improved 22 L packed-bed performance in comparison to 1.5 L or tray performances. This gives higher relevance to a correct $AFP_R$ adjustment when working with BB, in agreement with BB PCA analysis results in Chapter 5. In contrast, TH conidia production was more equal between different reactor configurations when looking at the same substrate. TH conidia production has overall been superior to BB's, suggesting better use of the substrate by this fungal strain. As previously found in Table 7.7 results, this analysis is consequent with obtained results in TH PCA analysis in Chapter 5, where parameters related to the biodegradability of the substrate were the most relevant for TH conidia production. This behaviour might also be attributed to the superior enzymatic production capabilities of TH (Verma et al., 2007) in comparison to BB. Aside from chitinases and as stated in previous chapters, the genera *Trichoderma* has been previously used to produce several enzymes, most of them being lignocellulosic enzymes such as cellulases, xylanases and endoglucanases (Lopez-Ramírez et al., 2019; Kar et al., 2013; Ortiz et al., 2015; Ahmed et al., 2016), whereas there are no reports on the use of BB to produce similar enzymes.

Achieved mean temperatures were similar among most of the reactors, presenting higher deviations in tray bioreactor than in packed bed and in beer draff than in rice husk. Although overall temperatures were a little bit lower when using rice husk, they were not always significantly different from beer draff. Most of the observed mean temperatures in all reactors and strains were around 25ºC. Interestingly, lowest mean temperature with both strains was observed in PBBs. Considering the thickness of the 22 L packed bed reactor in comparison to one tray bed's height (40 vs 4 cm), obtaining similar mean temperatures in both designs with both strains opens scaling-up possibilities for packed-beds. At the same time, higher bed thickness should also be tested for tray configurations, as demonstrated by several authors in different SSF tray fermenters (Krishna, 2005; Xie

et al., 2013; Zhang et al., 2014). It is noteworthy to mention that tray bioreactor insulation capabilities were probably superior to PBB, as tray bioreactor was adapted from an incubator, meaning potential better heat insulation. However, PBB were almost full of substrate, whereas tray bioreactor had a huge dead volume in all fermentations, meaning generated heat per volume unit was higher in PBB than in tray.

## Final remarks

Successful conidia production has been achieved using both BB or TH in tray bioreactor configuration. Differences in conidia production between trays were shown when working with BB, although they were not present when working with TH. Chitinase analysis in tray bioreactors revealed different optimal production times for conidia and for chitinase production. Chitinase activity values were similar between strains. However, maximum chitinase production was 1.5-1.75 times higher in TH fermentation in trays 1 and 2. BB strain production was affected by little temperature and moisture variation between trays, while TH overall performance was more similar between trays despite experimenting higher variations between trays for both parameters.

Higher productions with both strains were obtained when fermenting beer draff complemented with wood chips, which yielded higher conidia production in comparison to rice husk due to the use of a more easily biodegradable substrate with high $AFP_R$ while also obtaining much higher respiration profiles. BB performance was highly dependent on substrate $AFP_R$, while TH production was more dependent on substrate biodegradability, confirming PCA results presented in Chapter 5. While significant differences in terms of conidia production were shown between 22 L packed bed reactor inoculated with BB and the rest, these differences were not observed when working with TH, suggesting TH as a more versatile strain than BB. Total conidia production was similar between tray and 22 L PB when using same substrate. Although most reactors did not present significant differences in terms of mean temperature, better comparison could be made if adapting tray bed's thickness while also providing better heat insulation in 22 L PBB.

# Chapter 8

## Product validation and biocontrol potential

The experiments presented in this Chapter have been performed at the Department of Plant and Environmental Sciences, section of organismal biology (SOBI) at the University of Copenhagen (KU, Denmark), being part of a three months' research stay.

## 8.1. Summary/Overview

In this chapter, evaluation of the biopesticide effect presented by some of the BB SSF products obtained in previous Chapters is presented. Differences in biopesticide properties between BB and TH have been highlighted in Chapter 1: BB is an entomopathogenic fungi that can act as insect parasite and TH is an antagonistic fungus capable of numerous effects.

To properly evaluate biopesticide effect in entomopathogenic fungi, pathogenicity and virulence concepts are of major relevance. Pathogenicity is defined as the quality or state of being pathogenic, while virulence is the degree of pathogenicity within a group or species. Pathogenicity is a qualitative term, whereas virulence is the quantification of pathogenicity (Shapiro-Ilan et al., 2005). Both terms are commonly used when working in insect pathology. *T. molitor* has been chosen as pest to test BB against, as it is widely used as model host for the study of pathogenic fungi (de Souza et al., 2018). Its larvae stage, commonly known as mealworm, is a pest of storage grains and bran (Cotton, 1963; Philips and Throne, 2010). First report on the use of *T. molitor* in the investigation of fungal diseases dates to 1973 (Reiss, 1973). As shown in Figure 8.1, research on this pest has not stopped growing since then, reaching more than 2500 documents in recent Scopus search, showing higher growth in last decade and particularly from 2015 onwards.

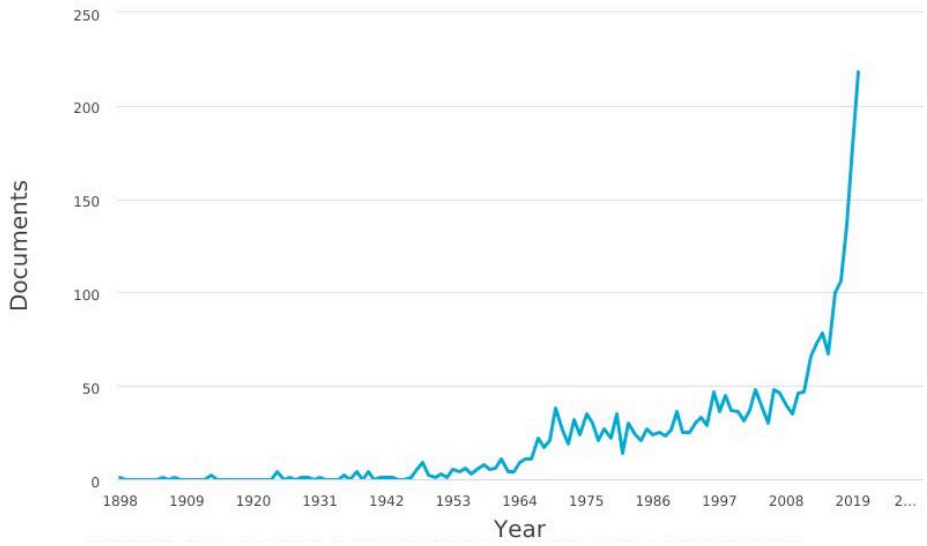



**Figure 8.1.** *T. molitor* research evolution from 1898 to 2021. Obtained from Scopus in August 2021.

The aim of this Chapter is to evaluate the biopesticide effect of the obtained BB product from 22 L reactors in Chapter 6, providing quantitative comparison on quality of conidia obtained from different biodegradability substrates.

## 8.2. Materials

Lyophilized BB material (conidia extracted from plates or solid material with conidia) was brought to Copenhaguen University (KU) to quantify its biopesticide properties. Samples obtained from plates and from various SSF tests presented in Chapter 6 were tested. The complete list of samples is presented in Table 8.1. Samples from 22 L SSF tests were obtained by mixing at least 3 samples from different reactor areas (superior, central and lower, as presented in Chapter 3). Samples treatment after fermentation has been detailed in section 3.3.7.1.

**Table 8.1.** Lyophilized samples used in biocontrol potential tests.

| Sample | Conidia (conidia $g^{-1}$dm) (before lyophilisation) | Conidia (conidia $mL^{-1}$) (before lyophilisation) | Conidia (conidia $mL^{-1}$) (after rehydration) |
|---|---|---|---|
| **BB plate** | (-) | $9.0x10^7$ | $7.3x10^7$ |
| **BB RH 22 L** | $6.0x10^8$ | $5.4x10^7$ | $4.8x10^7$ |
| **BB BD 22 L** | $2.5x10^9$ | $2.8x10^8$ | $1.1x10^8$ |
| **BB BD 22L (WV 10 L)** | $1.4x10^9$ | $1.9x10^8$ | $9.5x10^7$ |

BB: *Beauveria bassiana*; RH: rice husk; BDr: beer draff; WV: working volume; B: batch.

## 8.3. Tests

All tests in this Chapter were run for 14 days. Mycosis (fungal infection) of each cadaver was visually evaluated 4-6 days after dead of each individual by means of fungal emerging from the insect's dead body. Accumulated dead was used to calculate survivability for all the evaluated sample groups on each day.

BB Isolate KVL 13_39 was used as positive control in all tests. This isolate (obtained from the commercial biocontrol product BotaniGard containing the BB strain GHA) had been previously used in the SOBI research group and recognized to present high virulence against *T. molitor* (Tall and Meyling, 2018).

### 8.3.1. Dose-response tests

For testing the effects of different concentrations of the BB conidial suspensions from plate samples, a dose-response assay was performed using both larvae and adults of *T. molitor*. The assays were performed in triplicate with 10 individuals for each concentration. According to several authors (Seid et al., 2019; Maistrou et al., 2020; Yang et al., 2018; Oreste et al., 2012), intermediate virulent doses of BB against T. molitor range between $10^5$-$10^7$ conidia $mL^{-1}$ with droplet topical applications. Using BB conidia harvested from agar media plates, four concentrations of $10^5$, $10^6$, $10^7$, $10^8$ conidia $mL^{-1}$ were tested against *T. molitor* larvae and adult. Triton X 0.05% suspension was used as control and diluent. When performing assays with adults, only $10^5$, $10^7$ and $10^8$ concentrations of the CECT 20374 strain were tested. Schematic representation of the tests is shown in Figure 8.2.

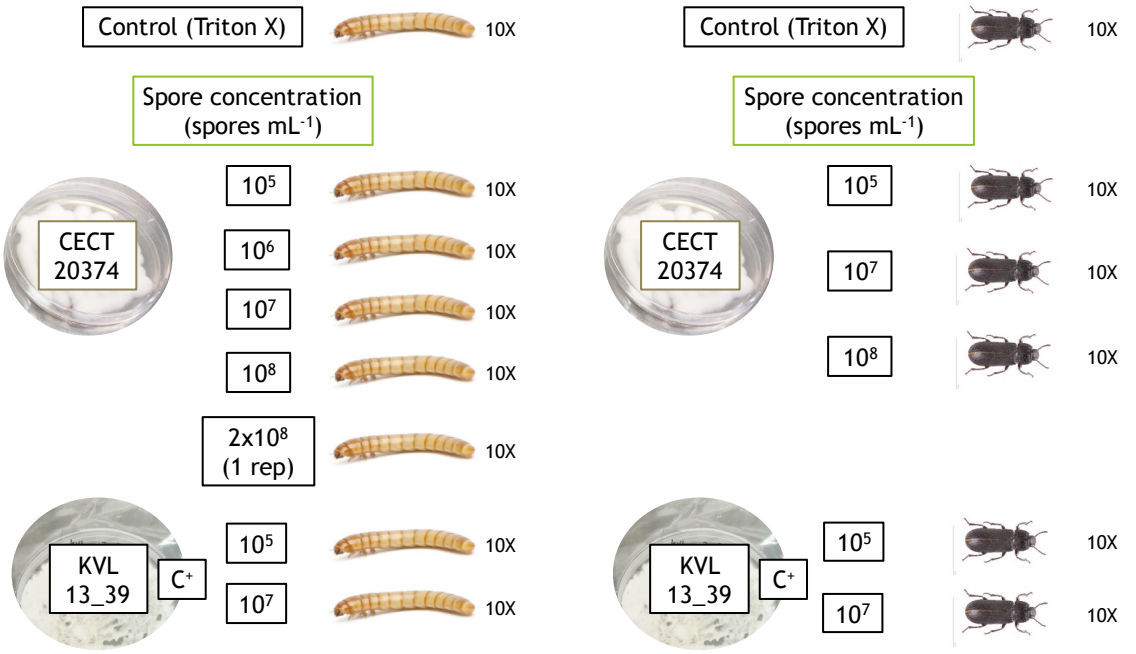



**Figure 8.2.** Schematic representation of BB concentration tests. Left: larvae assay. Right: adult assay.

### 8.3.2. Comparative virulence test

Based on the dose-response assays, an experiment to compare virulence of conidia obtained from different substrates against *T. molitor* was performed. As described by some authors (Keyser et al., 2016; Seid et al., 2019) virulence assays were performed using two concentrations, low (X1) and high (X2), respectively, which were previously

found to be in the lower and higher range of mortality in the dose-response assays in 8.3.1. The virulence assay was performed with 12 adult individuals for each concentration in triplicates. Schematic representation of the test is shown in Figure 8.3.

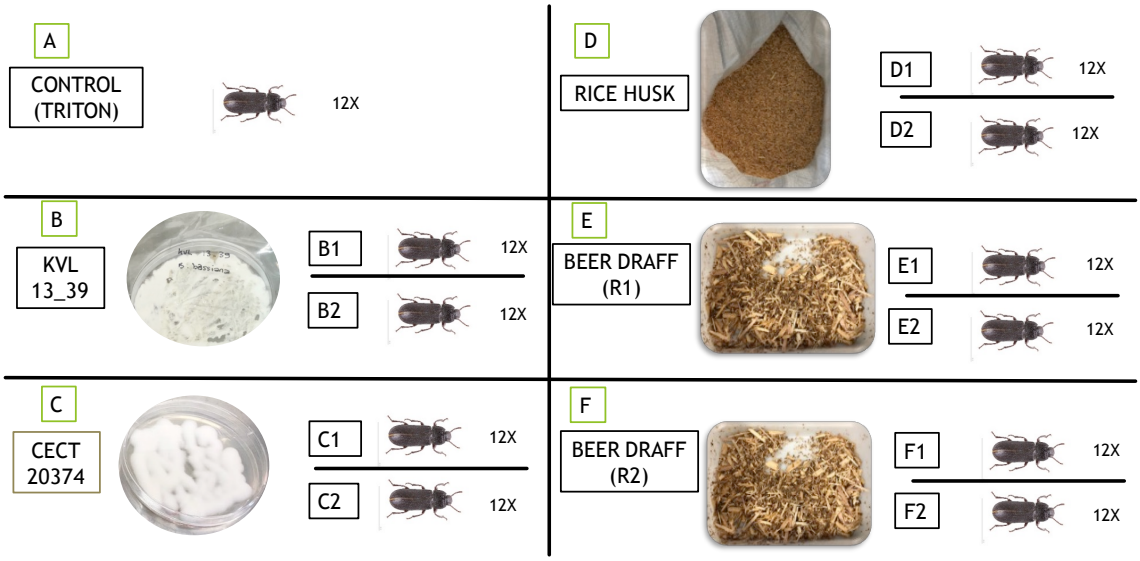



**Figure 8.3.** Schematic representation of BB virulence test.

## 8.4. Results and discussion

Germination test results for all samples used in this section are presented in Table 8.2.

In all total mortality graphs, statistical difference between total mortality is shown in black and statistical difference between mycosis in red. Control population mortality is also presented, despite using Abbott's formula to correct mortality on the rest of the samples.

**Table 8.2.** Germination test results for all samples.

| Sample | Germination (%) |
|---|---|
| KVL 13-39 | 95-97 [a] |
| CECT 20734 | 84-86 [b] |
| RH 22 L (CECT-SSF) | 63-67 [c] |
| BDr 22 L (CECT-SSF) | 74-77 [d] |
| BDr 22L (WV 10 L) (CECT-SSF) | 72-76 [d] |

RH: rice husk; BDr: beer draff; WV: working volume, SSF: solid-state fermentation. Stastical significance between tests is shown in superscript.

### 8.4.1. Dose-response tests

Figure 8.4. presents larvae *T. molitor* dose-response test results. Tests were performed with conidia suspension obtained from plate pure cultures. Total mortality and mycosis are shown in a) and survivability in b).

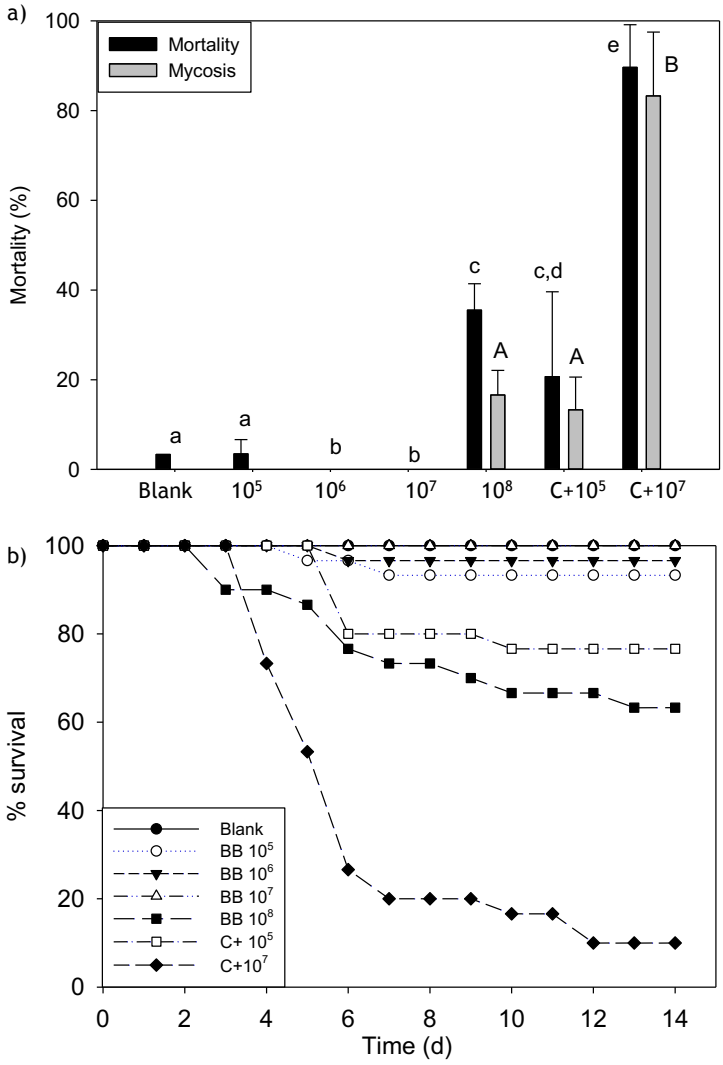



**Figure 8.4.** Larvae *T. molitor* concentration test results. a) total mortality and mycosis and b) survival evolution. BB samples correspond to CECT 20374 strain (used in this thesis) and C+ correspond to KVL 13_39.

Larval mortalities were generally very low for most of the tested concentrations below $10^8$. In contrast, the positive control treatments using BB GHA (strain KVL 13-39) produced significantly high mortality in a dose-dependent manner, ranging from 20 to 90% mortality between the two tested concentrations. Mortality in the lowest KVL 13-39 concentration was similar to mortality produced by the highest concentration of BB

CECT 20374. The proportion of mycosis development was also low for CECT 20374, around half of the dead population in $10^8$ conidia $mL^{-1}$ treatment, similar values as in lower concentration applied of KVL 13-39. The only exception was high concentration of KVL 13-39, which reached up to 83% mycosis of the total population, considered high mortality in other works testing other BB strains against *T. molitor* (Göttig and Hertz, 2018; Al Khoury et al., 2019; Seid et al., 2019). Additionally, this treatment was the only one presenting statistical significant differences with the other treatments. Different survival curve was also observed in the high concentration treatment of KVL 13-39, as survival decreased much faster (both in terms of time and slope) for this treatment than in any other treatment.

As shown in Table 8.2, differences in germination rates between the two BB strains were observed (being 10% lower in CECT 20374 strain in comparison to KVL 13-39), supporting the better performance of the KVL 13-39 strain. However, it would not be correct to assume germination index as the only explanation factor for the difference in infection performance between the two strains.

Results obtained both for mortality and mycosis suggest that strain CECT 20374 is not particularly virulent towards the *T. molitor* larval stage. Some studies have found the adult stage of *T. molitor* to be more susceptible to BB infection in comparison to larvae (Rodríguez-Gómez et al., 2009; Seid et al., 2019). Differences in structure of the cuticle between larvae and adults, as well as possible shedding of attached conidia on the cuticle after larvae ecdysis (Hajek and Leger, 1994; Vestergaard et al., 1999) might be the principal causes of differences in susceptibility within the same insect species. Consequently, adults of *T. molitor* were also tested as hosts for the two BB strains. Figure 8.5 presents adult *T. molitor* dose-response test results. Total mortality and mycosis are shown in a) and survivability in b).

Contrary to the larval stage, total mortality caused by strain CECT 20374 in adult *T. molitor* increased with increasing conidia concentration, starting at 40.7% for $10^5$ conidia $mL^{-}1$ and rising to 61 and 83% for $10^7$ and $10^8$ conidia $mL^{-1}$, respectively. This increase was also observed for level of mycosis, reaching its maximum when using the highest tested concentration, corresponding to nearly 60% of the total dead population. Despite these results, virulence was still not comparable to that of strain KVL 13-39, which achieved 100% dead adults at $10^7$ conidia $mL^{-1}$, while mortality similar to those caused by the CECT 20374 strain at both $10^7$ and $10^8$ conidia $mL^{-1}$ (around 80%), was reached already at $10^5$ conidia $mL^{-1}$ for KVL 13-39. Observed levels of mycosis were

similar between the highest CECT 20374 concentration and the lowest concentration of KVL 13-39 ($10^5$ conidia $mL^{-1}$), but mycosis level was highest for the high concentration of KVL 13-39, reaching values close to 80% of the total dead population.

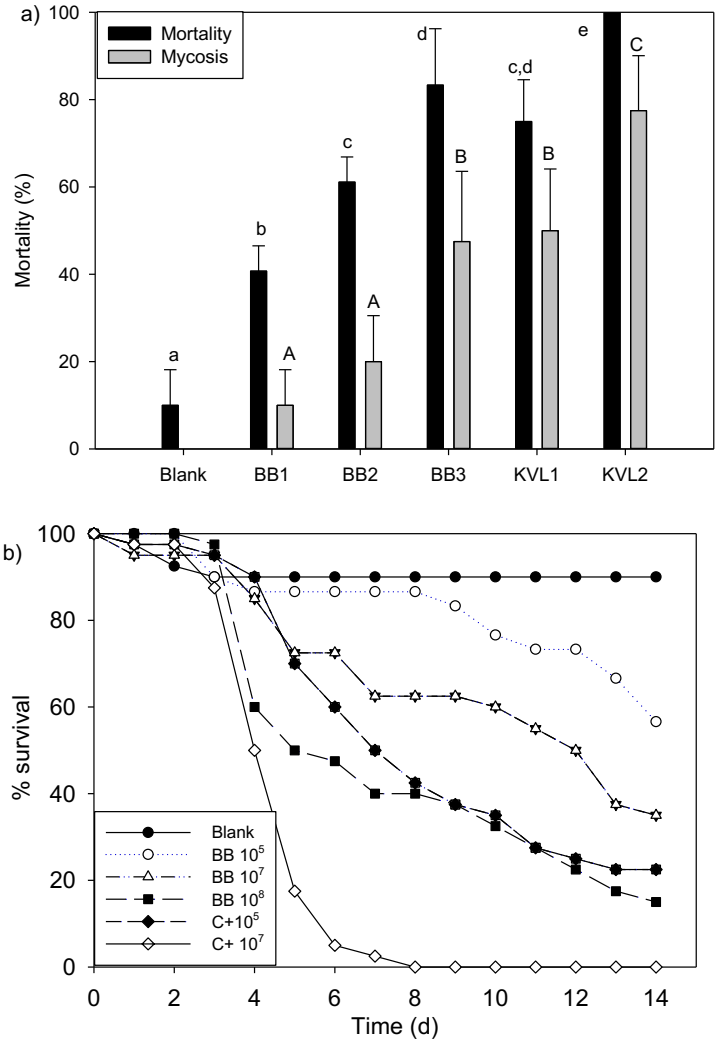



**Figure 8.5.** Adult T. *molitor* concentration test results a) total mortality and mycosis and b) survival evolution. BB samples correspond to CECT 20374 strain (used in this thesis) and C+ correspond to KVL 13_39.

Lower virulence of CECT 20374 strain is further evident from the survivorship curves (Fig. 8.5 b). Although the main mortality effects occur at day 4 for both $10^8$ conidia $mL^{-1}$ of CECT 20374 and $10^7$ conidia $mL^{-1}$ of KVL 13-39, the curves reach different percentages of survivors. At day 8, all individuals were dead in the treatment with $10^7$ conidia $mL^{-1}$ of KVL 13-39, whereas approximately 40% remained alive after the same period in the treatment with CECT 20374 despite a 10-fold higher exposure of conidia. These differences can partially be explained by reduced germination rate for the CECT

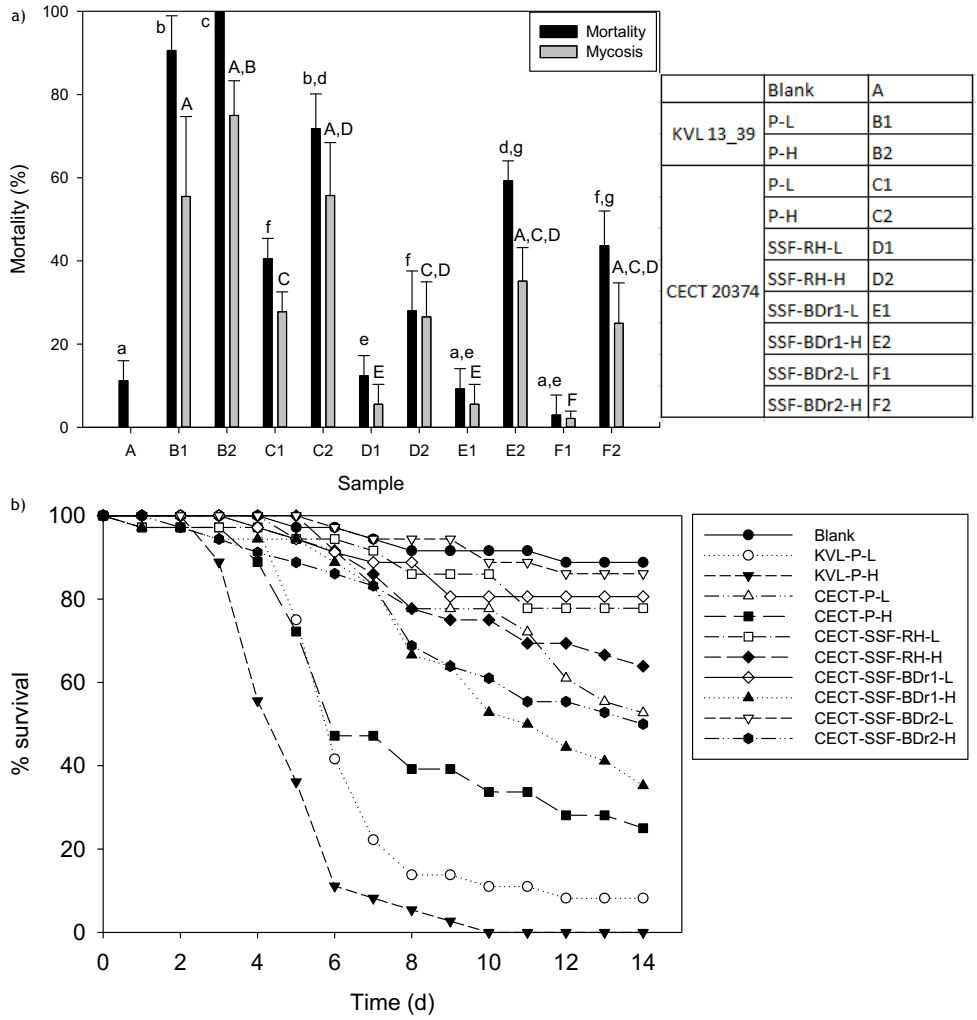
20374 strain, but are also reflecting reduced ability to infect *T. molitor* adults compared to KVL 13-39.

However, the CECT 20374 strain showed more pronounced virulence against the adult stage of T. molitor compared to the larval stage at the highest tested concentrations, but only at level equal to the low concentration used of the KVL 13-39 strain. Although the obtained virulence was still relatively low for CECT 20374 against the adults of *T. molitor*, mortality increased in a concentration dependent manner, confirming dose-dependent virulence against the insect.

### 8.4.2. Comparative virulence test

Virulence assay results against adult *T. molitor* are presented in Figure 8.6. Conidia from fermented samples CECT 20374 (BB-SSF) were compared with conidia from agar plates of the strains KVL 13-39 (C+) and CECT 20374 (BB). Both strains were tested at a low concentration of $10^6$ conidia $mL^{-1}$ and a high concentration of $5x10^7$ conidia $mL^{-1}$ for this comparative test.

Interestingly, differences in total mortality were observed depending on the substrate used to produce fungal conidia (Fig. 8.6a). At the high concentration, mortality of conidia from beer draff was higher than when using conidia produced on rice husk, being significantly different in one of the presented reactors (SSF-BDr1-H sample vs SSF-RH-H sample). Sample E2 (beer draff high conidia concentration) did not present significant differences with sample C2 (high concentration of CECT 20374 extracted from agar plates), which is considered to provide the optimal growth conditions for conidia. No differences were observed when comparing the low concentration for the two substrates, as little mortality with very low mycosis was obtained with all treatments. Except for E2, conidia quality loss was observed when comparing any sample to its correspondent agar plate control, which could also be expected from the germination test results in Table 2. These losses in viability were possibly caused by a sub-optimal downstream and conservation process. In fact, the downstream process was simple, consisting only of filtration and centrifugation steps with no formulation involved. These steps allowed conidia separation from almost all the solid material. However, accurate and complete formulation processes should be considered. As of conservation, all samples were frozen at -20ºC after the fermentation ended, unfrozen only to be lyophilised and after this kept at 4ºC for less than 5 days before its use after rehydration.




| | | |
|---|---|---|
| | Blank | A |
| KVL 13_39 | P-L | B1 |
| | P-H | B2 |
| CECT 20374 | P-L | C1 |
| | P-H | C2 |
| | SSF-RH-L | D1 |
| | SSF-RH-H | D2 |
| | SSF-BDr1-L | E1 |
| | SSF-BDr1-H | E2 |
| | SSF-BDr2-L | F1 |
| | SSF-BDr2-H | F2 |

**Figure 8.6.** Adult *T. molitor* virulence test results a) total mortality and mycosis and b) survival evolution. P indicates plate samples (KVL or CECT strains), SSF indicates fermentation samples (CECT strain). Low concentration (L) corresponds to $10^6$ conidia $mL^{-1}$, high concentration (H) corresponds to $5x10^7$ conidia $mL^{-1}$.

When lyophilising, conidia must be dried to a water content below 9% w/w or equal or to water activity equal to or lower than 0.3% to optimize shelf-life and maintain viability (Jaronski and Jackson, 2012; Moore et al., 2016). Drying and anaerobic packaging are required to maximize the storage period (Chen et al., 2009; Faria et al., 2012). Despite using recommended conservations methods, possible quality loses might still be attributed to sample conservation. Formulation is a crucial aspect of any biopesticide product development, highly affecting both viability and infectivity of the active ingredient (Mascarin and Jaronski, 2016; Jaronski, 1997; Brar et al., 2006). As such, both conidia extraction, separation and formulation should be improved to obtain maximal conidia virulence.

Sample appearance after mycosis occurred is shown in Figure 8.7.

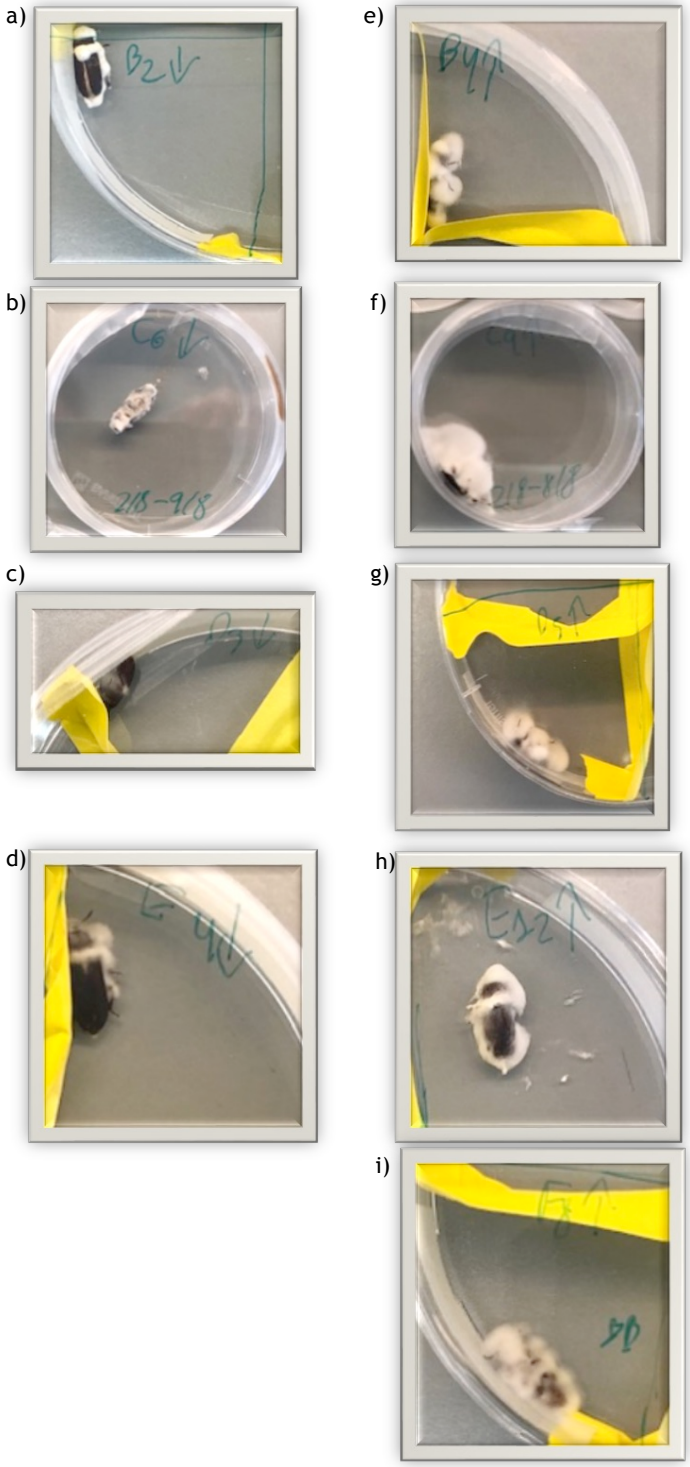



**Figure 8.7.** Examples of insect mycosis observed in dead *T. molitor* in virulence test. Left samples correspond to $1x10^6$ conidia $mL^{-1}$ and right samples to $5x10^7$ conidia $mL^{-1}$ a) KVL-P-L; b) CECT-P-L; c) CECT-SSF-RH-L; d) CECT-SSF-BDr1-L; e) KVL-P-H; f) CECT-P-H; g) CECT-SSF-RH-H; h) CECT-SSF-BDr1-H and i) CECT-SSF-BDr2-H.

Virulence differences between conidia from agar plates and fermentation samples can also be attributed to different conidia germination rates. While germination rates for conidia of strain CECT 20374 from agar plates were 84-86%, germination rates were 72-77% for conidia produced in beer draff samples and even lower for conidia from rice husk, reaching 63-67%. Although both substrates have been successful at producing BB conidia using a batch strategy, germination rates of conidia were higher when using beer draff compared to rice husk (Table 8.2). Consequently, higher virulence of conidia from beer draff samples could be expected. Despite this, there were no significant differences between observed mycosis in the high concentration samples, regardless of the substrate used.

Conidia produced in all fermentation samples were generally causing lower mortality than the typical values obtained in virulence assays with BB, which normally reach levels close to 90-100% at high concentrations (Keyser et al., 2014; Barta, 2018; Seid et al., 2019). High germination rates are mandatory to ensure proper infection and mycosis of the insect in such controlled experiments. All germination rates in the present study were calculated at 24 h of incubation. With the low percentages observed, and also considering growth and conidiation time of strain CECT 20374, it would be advisable to perform conidia germination tests at 36-48 h instead of 24 h, as presented in other works (Keyser et al., 2016).

When comparing treatments in the survivorship curves, mortality rates were higher and occurred earlier for conidia produced on agar plates for both strains in comparison to all fermentation samples. In most of these agar plate controls, most reduction in survival occurred between days 3-8 (KVL 13-39 at $10^6$ conidia $mL^{-1}$and CECT 20374 $5x10^7$ conidia $mL^{-1}$), even faster for KVL 13-39 at $5x10^7$ conidia $mL^{-1}$. In fermentation samples E2 and F2, the main reduction in survival occurred between days 5 and 10, being later than in agar plate controls. Formulation improvement should be directed not only at increasing total mortality but also to make mortality occur earlier, as seen for the same strain with conidia from agar plates.

$LT_{50}$ values corresponding to all samples that reached 50% survivability are presented in Table 8.3. Only four samples (KVL-P-L, KVL-P-H, CECT-P-H and SSF-BDr1-H) reached 50% survivability, although it was close in sample SSF-BDr2-H. As Statistical difference between $LT_{50}$ values confirm higher virulence of KVL 13-39 compared to CECT 20374, while also exemplifying virulence loss from plate to fermentation samples in CECT 20374 strain. Differences between plate and fermentation

virulence suggest fermentation step as a possible cause of viability losses. Differences in relevant parameters (such as temperature), being much easier to maintain close to optimum values when working with plates in comparison to reactors, could be the reason of viability losses. Nutrient availability differences could also explain lower values obtained with rice husk, as beer draff presented much higher biodegradability. Improvements in fermentation with the aim of maximizing virulence maintenance and not only conidia production should be considered.

**Table 8.3.** $LT_{50}$ values for samples with at least 50% survaivability.

| Sample | $LT_{50}$ (d) |
|---|---|
| KVL-L | 5.8 |
| KVL-H | 4.4 |
| CECT-P-H | 7.2 |
| CECT-SSF-H, BDr | 10.7 |

$LT_{50}$: lethal time 50; BDr: beer draff; L: low concentration ($10^6$ conidia $mL^{-1}$); H: high concentration ($5x10^7$ conidia $mL^{-1}$); P: plate; SSF: solid-sate fermentation

## Final remarks

Virulence effect of the BB SSF 22 L reactor products using rice husk or beer draff as substrate was successfully tested against *T. molitor* adult stage, presenting a biopesticide effect against this insect in laboratory assays. Differences in virulence depending on the fermentation substrate used to produce fungal conidia were detected, with beer draff being most suitable. Relevance of formulation technology was highlighted, emphasizing in its relevance to maintain product virulence after fermentation. Differences observed between plate and fermented samples also suggest fermentation step as a possible explanation for quality losses, highlighting the need to optimize it in order to maximize virulence maintenance.

# Chapter 9

Conclusions

## 9.1. General conclusions

- Fungal conidia production of BB and TH in PBB using different autoclaved agro-industrial wastes as substrates has been achieved.
- Fungal SSF fermentation parameters with both strains have been optimized at 0.5 L laboratory scale using rice husk as substrate. Relevance of several parameters on fungal SSF have been studied, allowing the selection of suitable substrates for conidia production.
- Valorisation of the studied residues (rice husk, apple pomace, whisky draff, wheat straw, beer draff, orange peel and potato peel) through SSF has been demonstrated feasible, highlighting the main waste properties and process parameters for the two fungi used.
- A robust, reproducible, and scalable process to produce BB and TH conidia in SSF packed bed bioreactors using substrates of different biodegradability has been achieved. Beer draff has been determined as preferable over rice husk.
- Promising results were obtained when scaling the process up to 22 L, particularly when using TH, allowing the use of a SBR strategy when working with beer draff complemented with wood chips as substrate.
- PBB performance was comparable to tray performance using same substrate with both strains.
- Virulence (BB) properties were present in the final fermentation products.

## 9.2. Block 1: SSF validation, optimization and substrate screening

- In SSF process optimization tests using rice husk, optimized parameters at 0.5 L scale for BB were 65-70% moisture, $5.5x10^6$ conidia $g^{-1}$dm inoculum concentration, 20 mL/min airflow, 25ºC temperature and 40 C/N ratio. Same values were obtained with TH except for moisture (55-60%) and C/N ratio (25-55). Mixing was positive to TH conidia production when performed at 24 h or 48 h after inoculation.
- The robustness of the process shown through Box-plots allows establishing a highly probable conidia production range valid both for BB and TH ($5.0x10^8$-$1.3x10^9$ conidia $g^{-1}$dm).

- In substrate screening tests, rice husk, apple pomace, whisky draff, beer draff, wheat straw, orange peel and potato peel have been successful substrates for fungal conidia production for both strains. Soy fiber and rice fiber were discarded due to bacterial contamination.
- Rice husk and potato peel were more suitable for BB whereas beer draft, orange peel and potato peel are more suitable for TH.
- According to PCA, relevant BB SSF parameters (initial pH and $AFP_R$) link to proper adaptation to the substrate to ensure fungal growth, while relevant TH parameters (COC, initial moisture and total sugar content) relate to potential substrate biodegradability.

## 9.3. Block 2: production strategies and scale-up to 22 L

- Process scale-up to 22 L has been successful using both rice husk or beer draff and wood chips as substrates. For BB, batch strategy has been the preferable choice due to presence of unavoidable AN contamination, reaching conidia production of $2.5x10^9$ conidia $g^{-1}$dm. For TH, SBR strategy has been successful using the mixture of beer draff and wood chips, sustaining conidia production for 5 consecutive batches at values close to $2.0x10^9$ conidia $g^{-1}$dm.
- Beer draff has been preferred as substrate due to presenting less contamination (AN) than rice husk.
- $AFP_R$ has been determined as a key parameter in process scale-up thanks to observed differences between 1.5 and 22 L. A minimum $AFP_R$ value of 80% has been defined as necessary for the correct scaling of the process.
- Process robustness was demonstrated with packed bed uniformity in all 22 L reactors with both substrates and strains, despite their different biodegradability. Relevant differences were only found for temperature when using beer draff as substrate due to its superior biodegradability potential.
- SBR strategy has been demonstrated as a feasible alternative to traditional batch operation, at least up to a scale of 22 L.
- Successful conidia production has been achieved using both BB and TH in tray bioreactor configuration. Differences in conidia production between trays were observed with BB, while differences in chitinase activity were observed in TH.

- Conidia production comparison in different reactor configurations confirmed PCA results presented in Chapter 5 both for BB and TH.
- TH was demonstrated as a more versatile strain when compared to BB, as no differences in conidia production were observed among different reactor configurations.

## 9.4. Block 3: product validation and biocontrol potential

- Virulence of the produced BB strain against the pest *T. molitor* has been demonstrated. Virulence was only possible against adult stage of the pest.
- Higher virulence was found on beer draff fermentations product when comparing to rice husk, highlighting substrate influence on quality of the final product.
- Virulence loss comparing to plate samples suggest the need of improvements in fermentation, downstream, conservation and formulation.

## 9.5. Future work

Being the first work in GICOM to attempt to produce fungal conidia via SSF and given the scarcity of research on the use of packed bed reactors to produce fungal conidia, future work is still to be performed:

- Beer draff should be used to scale-up the process in PBBs up to higher volumes (at least to preindustrial scale). SBR strategy has potential to be used at higher scales, and should therefore be tested, at least with TH.
- Future tests should also focus on analyzing enzymatic activities of the fermentation products as an indicator of biocontrol potential, particularly chitinases but also considering other possibilities. These analyses have yet to be performed in packed bed configuration and could be related to strain virulence against pests.
- For BB, strains presenting high virulence (like KVL 13_39) should be tested and produced. Fermentation strategies presented in this thesis can be used to produce them. The use of potential strains presenting high entomopathogenic capabilities should lead to maximization of the biocontrol potential of the final product.
- For TH, obtained product from both substrates should be tested, with the objective of demonstrating if its capabilities (antagonistic/biostimulant, etc.) are maintained

during successive SBR fermentations. This assays should be conducted not only at laboratory scale but also in greenhouse and/or field.

- This thesis has been focused on the fermentation process. Consequently, a significant effort should be made both in downstream and formulation for both strains' products but specially with BB, as necessary steps to maximize its biocontrol potential.

# Chapter 10

References

# 10. References

Abubaker, A., Mohamed, I., Abdalla, F., Adam, M., Abdelkreim, M., Gammareldain, A., 2015. Analysis of soil NPK, pH and electrical conductivity at Adham area- renk, upper Nile state. Int. J. Sien. Tech. Res. 4 (12), 341–347.

Aguilar-Zárate, P., Wong-Paz, J.E., Rodríguez-Duran, L.V., Buenrostro-Figueroa, J., Michel, M., Saucedo-Castañeda, G., Favela-Torres, E., Ascacio-Valdés, J.A., Contreras-Esquivel, J.C., Aguilar, C.N., 2018. On-line monitoring of *Aspergillus niger* GH1 growth in a bioprocess for the production of ellagic acid and ellagitannase by solid-state fermentation. Bioresource Technol. 247, 412-418.

Ahmed, I., Zia, M.A., Iqbal, H.M.N., 2016. Detergent-compatible purified endoglucanase from the agro-industrial residue by *Trichoderma harzianum* under solid state fermentation. BioResources. 11(3), 6393-6406.

Aita, B.C., Spannemberg, S.S., Schmaltz, S., Zabot, G.L., Tres, M.V., Kuhn, R.C., Mazutti, M.A., 2019. Production of cell-wall degrading enzymes by solid-state fermentation using agroindustrial residues as substrates. J. Environ. Chem. Eng. 7, 103-193.

Al Khoury, C., Guillot, J., Nemer, N., 2019. Lethal activity of beauvericin, a *Beauveria bassiana* mycotoxin, against the two-spotted spider mites, *Tetranychus urticae* Koch. J. Appl. Entomol. 9, 974-983.

Aliyu, S., Bala, M., 2011. Brewer's spent grain: a review of its potentials and applications. African J. Biotechnol. 10, 324–331.

Allen, M.T., Levi, L.S., 2013. Parkinson's disease and pesticide exposure – a new assessment. Crit. Rev. Toxicol. 43(6), 515-534.

Anand, S., Reddy, J., 2009. Biocontrol potential of Trichoderma sp. against plant pathogens. Int. J. Agr. Sci. 1, 30-39.

Arzumanov, T., Jenkins, N., Roussos, S., 2005. Effect of aeration and substrate moisture content on sporulation of *Metarhizium anisopliae* var. acridum. Process Biochem. 40(3–4), 1037–1042.

Aydin, A., Aksu, H., Gunsen, U., 2011. Mycotoxin levels and incidence of mould in Turkish rice. Environ. Monit. Assess. 178, 271-280.

Balakrishnan, S., Srikanth, J., Santhalakshmi, G., Hari, K., Sankaranarayanan, C., 2011. Response of the entomopathogenic fungi *Beauveria Bassiana* and *Metarhizium*

*Anisopliae* to molasses media fortified with supplements. J. Sugarcane Research. 1,57–65.

Balasubramanian, S., Tyagi, R.D., 2017. Biopesticide production from solid wastes. Current Developments in Biotechnology and Bioengineering: Solid Waste Manage. 43, 58.

Ballardo, Cindy Vanessa., 2016. Valorización de residuos sólidos orgánicos como sustrato para el crecimiento de Bacillus thuringiensis mediante fermentación en estado sólido: aplicación a la fracción orgánica de residuos municipales para la producción de compost con efecto biopesticida. PhD thesis. Universitat Autònoma de Barcelona.

Ballardo, C., Barrena, R., Artola, A., Sánchez, A., 2017. A novel strategy for producing compost with enhanced biopesticide properties through solid-state fermentation of bio- waste and inoculation with *Bacillus thuringiensis*. Waste Manage. 70, 53–58.

Barrena, R., Gea, T., Ponsà, S., Ruggieri, L., Artola, A., Font, X., Sánchez, A., 2011. Categorizing raw organic material biodegradability via respiration activity measurement: a review. Compost Sci. Util. 19, 105-113.

Barrera, M.C., Gómez, M.I., Bermúdez, J.C.S., 2019. Towards the production of fungal biocontrol candidates using inert supports: a case of study of *Trichoderma asperellum* in a pilot fixed bed fermenter. Biocontrol Sci. Techn. 29(2), 162-184.

Barta, M., 2018. In planta bioassay on the effects of endophytic *Beauveria* strains against larvae of horse-chestnut leaf miner (*Cameraria ohridella*). Biol. Control. 121, 88-98.

Bastidas, O., 2009. Technical note – Neubauer chamber cell counting. www.researchgate.net/publictopics.PublicPostFileLoader.html?id=53c6a939d4c11856478b45d2&key=89353f79-aa75–4e19-8161-d900619b728d (accessed November 2017).

Behie, S.W., Zelisko, P.M., Bidochka, M.J., 2012. Endophytic insect-parasitic fungi translocate nitrogen directly from plants. Science. 336, 1576–1578.

Berini, F., Katz, C., Gruzdev, N., Casartelli, M., Tettamanti, G., Marinelli, F., 2018. Microbial and viral chitinases: Attractive biopesticides for integrated pest management. Biotechnol. Adv. 36, 818-838.

Berna, K.S., Korkmaz, G.H., Akin, D.A., 2012. Production of a Thermostable and Alkaline Chitinase by Bacillus thuringiensis subsp. kurstaki Strain HBK-51. Biotechnol. Res. Int. Volume 2012, Article ID 135498. doi:10.1155/2012/135498.

Bernal-Vicente, A., Pascual, J.A., Tittarelli, F., Hernández, J.A., Diaz-Vivancos, P., 2015. *Trichoderma harzianum* T-78 supplementation of compost stimulates the antioxidant defence system in melon plants. J. Sci. Food Agric. 95, 2208–2214.

Bhanu Prakash, G.V.S., Padmaja, V., Siva Kiran, R.R., 2008. Statistical optimization of process variables for the large-scale production of *Metarhizium anisopliae* conidiospores in solid-state fermentation. Bioresource Technol. 99(6), 1530–1537.

Bhateria, R., Dhaka, R., 2019. Optimization and statistical modelling of cadmium biosorption process in aqueous medium by *Aspergillus niger* using response surface methodology and principal component analysis. Ecol. Eng. 135, 127–138.

Bolognesi, C., 2003. Genotoxicity of pesticides: a review of human biomonitoring studies. Mutation research – Reviews in Mutation Research. 543(3), 251-272.

Boomsma, J.J., Jensen, A.B., Meyling, N.V., Eilenberg, J., 2014. Evolutionary interaction networks of insect pathogenic fungi. Annu. Rev. Entomol. 59(1), 467–485.

Botella, C., De Ory, I., Webb, C., Cantero, D., Blandino, A., 2005. Hydrolytic enzyme production by *Aspergillus awamori* on grape pomace. Biochem. Eng. J. 26(2–3), 100 – 106.

Brar, S.K., Verma, M., Tyagi, R.D., Valéro, J.R., 2006. Recent advances in downstream processing and formulations of *Bacillus thuringiensis* based biopesticides. Process Biochem. 41(2), 323–342.

Bunbury-Blanchette, A.L., Walker, A.K., 2019. *Trichoderma* species show biocontrol potential in dual culture and greenhouse bioassays against *Fusarium* basal rot of onion. Biol. Control. 130, 127-135.

Capinera, J.L., 2004. Encyclopedia of Entomology. Kluwer Academic, Dordretch, Boston

Casciatori, F.P., Laurentino, C.L., Taboga, S.R., Casciatori, P.A., Thoméo, J.C., 2014. Structural properties of beds packed with agro-industrial solid by-products applicable for solid-state fermentation: Experimental data and effects on process performance. Chem. Eng. J. 255, 214-224.

Cavalcante, R.S., Lima, H.L.S., Pinto, G.A.S., Gava, C.A.T., Rodrigues, S., 2008. Effect of moisture on *trichoderma* conidia production on corn and wheat bran by solid state fermentation. Food and Bioprocess Technol. 1, 100–104.

Cerda, A., 2017. Sustainable carbohydrase production using organic wastes through solid-state fermentation: operational strategies and microbial communities assessment. PhD thesis. Universitat Autònoma de Barcelona.

Cerda, A., Mejías, L., Gea, T., Sánchez, A., 2017. Cellulase and xylanase production at pilot scale by solid-state fermentation from coffee husk using specialized consortia: The consistency of the process and the microbial communities involved. Bioresour. Technol. 243, 1059–1068.

Chang, Y. L. and Wang, J. H., 2018, June. A study of the optimal condition for the growth of *Aspergillus niger* (BCRC 31494, ATCC 10864). Poster presented at BEST Conference & International Symposium on Biotechnology and Bioengineering (BEST 2018) At: Taipei, Taiwan.

Cheirsilp, B., Kitcha, S., 2015. Solid state fermentation by cellulolytic oleaginous fungi for direct conversion of lignocellulosic biomass into lipids: Fed-batch and repeated-batch fermentations. Ind. Crop. Prod. 66,73–80.

Chelliappan, B., Madhanasundareswari, K., 2013. Production and optimization of growth conditions for invertase enzyme by *Aspergillus Sp*., in solid state fermentation (SSF) using papaya peel as substrate. J. Microbiol. Biotechn. Food Sci. 3(3), 266-269.

Chen, C., Wang, Z., Ye, S., Feng, M., 2009. Synchronous production of conidial powder of several fungal biocontrol agents in series fermentation chamber system. Afr. J. Biotechnol. 8, 3649–3653.

Chu, Y.T., Lo, C.T., Chang, S.C., Lee, T.T., 2017. Effects of Trichoderma fermented wheat bran on growth performance, intestinal morphology and histological findings in broiler chickens. Ital. J. Anim. Sci. 16(1), 82–92.

Copping, L.G., Menn, J.J., 2000. Biopesticides: a review of their action, applications and efficacy. Pest Manage. Sci. 56, 651 – 676.

Cotton, R.T., 1963. Pests of stored grain and grain products. Burgess Publishing Company: Minneapolis, Minnesota, USA. p. 318.

Couto, S.R., Sanromán, M.Á., 2006. Application of solid-state fermentation to food industry - A review. J. Food Eng. 76(3), 291–302.

da Cunha, L.P., Casciatori, F.P., Vicente, I.V., Garcia, R.L., Thoméo, J.C., 2020. *Metarhizium anisopliae* conidia production in packed bed bioreactor using rice as substrate in successive cultivations. Process Biochem. 97, 104-111.

Daryaei, A., Jones, E.E., Ghazalibiglar, H., Glare, T.R., Falloon, R.E., 2016. Effects of temperature, light and incubation period on production, germination and bioactivity of *Trichoderma atroviride*. J. Appl. Microbiol. 120(4), 999–1009.

De la Cruz Quiroz, R., Roussos, S., Hernández, D., Rodríguez, R., Castillo, F., Aguilar, C.N., 2015. Challenges and opportunities of the biopesticides production by solid-state fermentation: filamentous fungi as a model. Crit. Rev. Biotechnol. 35(3), 326–333.

De la Cruz-Quiroz, R., Robledo-Padilla, F., Aguilar, C.N., Roussos, S., 2017. Forced Aeration Influence on the Production of Spores by Trichoderma strains. Waste Biomass Valori. 8, 2263-2270.

De Souza, P.C., Caloni, C.C., Wilson, D., Almeida, R.S., 2018. An invertebrate host to study fungal infections, mycotoxins and antifungal drugs: *Tenebrio molitor*. J. fungi. 4, 125.

Desfarges, C., Larroche, C., Gros, J.B., 1987. Spore production of Penicillium roquefortii by solid state fermentation: Stoichiometry, growth and sporulation behavior. Biotechnol. Bioeng. 29, 1050-1058.

Dhar, P., Kaur, G., 2011. Response surface methodology for optimizing process parameters for the mass production of *Beauveria bassiana* conidiospores. Afr. J. Microbiol. Res. 4, 2399–2240.

Dhar, S., Jindal, V., Gupta, V.K., 2016. Optimization of growth conditions and medium composition for improved conidiation of newly isolated *Beauveria bassiana* strains. Indian J. Exp. Biol. 54, 634–643.

Dhillon, G., Kaur, S., Brar, S., Verma, M., 2012. Potential of apple pomace as a solid substrate for fungal cellulase and hemicellulase bioproduction through solid-state fermentation. Ind. Crops Prod. 38, 6–13.

Dorta, B., Arcas, J., 1998. Sporulation of *Metarhizium anisopliae* in solid-state fermentation with forced aeration. Enzyme Microb. Tech. 23(7–8), 501–505.

Echegaray, T., 2020. Diseño de un reactor de bandejas para la producción de biopesticidas fúngicos. Master thesis. Universitat Autònoma de Barcelona.

Evans, H.C., Hywel-Jones, N.L., 1997. Entomopathogenic fungi. In: Ben-Dov, Y., Hudgson, C.J., (eds). Soft scale insects - their biology, natural enemies and control, vol 7B. Elsevier, Amsterdam.

Faria, M.R., Wraight, S.P., 2007. Mycoinsecticides and mycoacaricides: a comprehensive list with worldwide coverage and international classification of formulation types. Biol. Control. 43:237–256.

Faria, M., Hotchkiss, J.H., Wraight, S.P., 2012. Application of modified atmosphere packaging (gas flushing and active packaging) for extending the shelf life of *Beauveria bassiana* conidia at high temperatures. Biol. Control. 61, 78–88.

Finkler, A.T.J., Weber, M.Z., Fuchs, G.A., Scholz, L.A., Luz Jr, L.F.L., Krieger, N., Mitchell, D.A., Jorge, L.M.M., 2021. Estimation of heat and mass transfer coefficients in a pilot packed bed solid-state fermentation bioreactor. Chem. Eng. J. 408, 127246.

Finney, D.J., 1971. Probit Analysis, Third Edition. London, Cambridge University Press.

Food and Agriculture organization of the United Nations (FAO). FAOSTAT: pesticides use. Available at: http://www.fao.org/faostat/en/#data/RP/visualize. Consulted: April 2021.

Food and Agriculture organization of the United Nations (FAO), 2020. FAOSTAT: Corporal Statistical Database. Crops/Regions/World Elements/Area harvested/Production quantity Items/rice (paddy) Years/Select all. Consuted: April 21. Link: http://www.fao.org/faostat/en/#data/QCL.

Fredlund, E., Thim, A.M., Gidlund, A., Brostedt, S., Nyberg, M., Olsen, M., 2009. Moulds and mycotoxins in rice from the Swedish retail market. Food Addit. Contam. A. 26, 527-533.

Gao, K.X., Liu, X.G., Liu, Y.H., Zhu, T.B., Wang, S.L., 2002. Potential of *Trichoderma harzianum* and *T.atroviride* to control *Botryosphaeria berengeriana* f. sp. piricola, the cause of apple ring rot. J. Phytopathol. 150: 271-276.

Gao, L., Sun, M.H., Liu, X.Z., Che, Y.S., 2007. Effects of carbon concentration and carbon to nitrogen ratio on the growth and sporulation of several biocontrol fungi. Mycol. Res. 111,87–92.

Garza-López, P.M., Konigsberg, M., Gómez-Quiroz, L.E., Loera, O., 2012. Physiological and antioxidant response by *Beauveria bassiana* Bals (Vuill.) to different oxygen concentrations. World J. Microb. Biot. 28(1), 353–359.

Ghori, M.I., Ahmed, S., Malana, M.A., Jamil, A., 2012. Kinetics of exoglucanase and endoglucanase produced by *Aspergillus niger* NRRL 567. Afr. J. Biotechnol. 11(28), 7227-7231.

Ghose, T., 1987. Measurement of cellulase activities. Pure Appl. Chem. 59, 257–268.

Gil-Serna, J., García-Díaz, M., Vázquez, C., González-Jaén, M.T., Patiño, B., 2019. Significance of *Aspergillus niger* aggregate species as contaminants of food products in Spain regarding their occurrence and their ability to produce mycotoxins. Food Microbiol. 82, 240-248.

Glare, T., Caradus, J., Gelernter, W., Jackson, T., Keyhani, N., Kohl, J., Stewart, A., 2012. Have biopesticides come of age? Trends Biotechnol. 30(5), 250–258.

González, I., Infante, D., Peteira, B., Martínez, B., Arias, Y., González, N., Miranda, I., 2010. Caracterización bioquímica de aislamientos de *trichoderma spp*. promisorios como agentes de control biológico y expresión de actividad quitinasa. Revista. Protección Vegetal. 25, 58-63.

Göttig, S., Herz, A., 2018. Susceptibility of the Box tree pyralid *Cydalima perspectalis* Walker (Lepidoptera: Crambidae) to potential biological control agents Neem (NeemAzal®-T/S) and entomopathogenic nematodes (Nemastar ®) assessed in laboratory bioassays and field trials. J. Plant Dis. Protect. 125, 365-375.

Hajek, A.E., Leger, R.J., 1994. Interactions between fungal pathogens and insect hosts. Annu. Rev. Entomol. 39, 293–322.

Hallsworth, J.E., Magan, N., 1996. Culture age, temperature, and pH affect the polyol and trehalose contents of fungal propagules. Appl. Environ. Microb. 62, 2435–2442.

Holder, D.J., Kirkland, B.H., Lewis, M.W., Keyhani, N.O., 2007. Surface characteristics of the entomopathogenic fungus *Beauveria (Cordyceps) bassiana.* Microbiology. 153(10), 3448–3457.

Hölker, U., Höfer, M., Lenz, J., 2004. Biotechnological advantages of laboratory-scale solid-state fermentation with fungi. Appl. Microbiol. Biot. 64, 175–186.

Hong-jun, L., Wan-dong, D., Chao, L., Ling-xue, M., Hong.xu, L., Rong, L., Qui-rong, S., 2021. Spore production in the solid-state fermentation of stevia residue by *Trichoderma guizhouense* and its effects on corn growth. J. Integr. Agr. 20(5) 1147-1156.

Humber, R.A., 2008. Evolution of entomopathogenicity in fungi. J. Invertebr. Pathol. 98(3), 262–266.

Humber, R.A., 2012. Chapter X: preservation of entomopathogenic fungal cultures. In: Lacey, L (ed). Manual of techniques in invertebrate pathology, pp. 317-328. ElSevier.

Ibarruri, J., Cebrián, M., Hernández, I., 2019. Solid state fermentation of brewer's spent grain using *Rhizopus sp*. to enhance nutritional value. Waste and Biomass Valori. 10, 3687–3700.

Inglis, G.D., Goettel, M.S., Butt, T.M., Strasser, H., 2001. Use of hyphomycetous fungi for managing insect pests. In: Butt, T. M., Jackson, C., Magan, N. (eds) Fungi as biocontrol agents. Progress, problems and potential. CABI Publishing, Wallingford, pp 23–69.

Inglis, G.D., Enkerli, J., Goettel, M.S., 2012. Laboratory techniques used for entomopathogenic fungi: Hypocreales. In: Lacey, L. A (Ed)., Manual of techniques in invertebrate pathology. IP Consulting International, Yakima, Washington, USA.

International Rice Research Institute (IRRI), 2020. Rice knowledge bank. Home/Step-by-step production/Postproduction/By-products/Rice husk. Consulted: April 21. Link:http://www.knowledgebank.irri.org/step-by-step-production/postharvest/rice-by-products#rice-husk.

Jaronski, S.T., 1997. New paradigms in formulating mycoinsecticides. Pesticide formulations and application systems. Am. Soc. Test. Mat. 17, 99–114.

Jaronski, S.T., Jackson, M.A., 2012. Mass production of entomopathogenic hypocreales. In: Lacey, L. (Ed.). Manual of techniques in invertebrate pathology (2nd ed.) (pp. 255–284). New York, NY: Academic Press.

Jaronski, S.T., 2014. Mass production of entomopathogenic fungi: state of the art. In J. Morales-Ramos, M.G., Rojas, D., Shapiro-Ilan (Eds.), Mass production of beneficial organ- isms. Invertebrates and enthomopathogens. Elsevier. 357–413.

Jaronski, S.T., Mascarin, G.M., 2017. Mass production of fungal entomopathogens. In: Lacey, L.A (ed). Microbial control of insect and mite pests. From theory to practice., pp 141-155. ElSevier.

Jenkins, N.E., Heviefo, G., Langewald, J., Cherry, A.J., Lomer, C.J., 1998. Development of mass production technology for aerial conidia for use as mycopesticides. BioControl. 19,21–32.

Jin, B., Zepf, F., Bai, Z., Gao, B., Zhu, N., 2016. A biotech-systematic approach to select fungi for bioconversion of winery biomass wastes to nutrient-rich feed. Process Saf. Environ. 103,60–68.

Jou, R.Y., Lo, C.T., 2011. Heat and mass transfer measurements for tray-fermented fungal products. Int. J. Thermophys. 32(1-2), 523–536.

Kar, S., Gauri, S.S., Das, A., Jana, A., Maity, C., Mandal, A., Mohapatra, P.K.D., Pati, B.R., Mondal, K.C., 2013. Process optimization of xylanase production using cheap solid substrate by *Trichoderma reesei* SAF3 and study on the alteration of behavioral properties of enzyme obtained from SSF and SmF. Bioprocess Biosyst. Eng. 36, 57-68.

Kelbert, M., Romaní, A., Coelho, E., Pereira, F.B., Teixeira, J.A., Domingues, L., 2015. Lignocellulosic bioethanol production with revalorization of low-cost agroindustrial by- products as nutritional supplements. Ind. Crop. Prod. 64,16–24.

Kemsley, E.K., Defernez, M., Marini, F., 2019. Multivariate statistics: Considerations and confidences in food authenticity problems. Food Control. 105, 102-112.

Keyser, C.A., Thorup-Kristensen, C., Meyling, N.V., 2014. *Metarhizium* seed treatment mediates fungal dispersal via roots and induces infections in insects. Fungal Ecol. 11, 122-131.

Keyser, C.A., Jensen, B., Meyling, N.V., 2016. Dual effects of *Metarhizium* spp. and *Clonostachys rosea* against an insect and a seed-borne pathogen in wheat. Pest Manag. Sci. 72, 517-526.

Krishania, M., Sindhu, R., Binod, P., Ahluwalia, V., Sangwan, R.S., Pandey, A., 2018. Design of bioreactors in solid-state fermentation. In: Pandey, A., Larroche, C., Soccol, C.R (Eds)., Current Developments in Biotechnology and Bioengineering: Current advances in Solid-state Fermentation. ElSevier. 83-96.

Krishna, C., 2005. Solid-state fermentation systems - An overview. Crit. Rev. Biotechnol. 25(1-2), 1–30.

Kubicek, C., Harman, G.E., 1998. *Trichoderma* and *Gliocladium* Volume 1 Basic biology, taxonomy and genetics. In: W. Gams, J. Bissett (eds.) Morphology and identification of *Trichoderma.* London: Taylor & Francis group. 3–34.

Lai, T., Su, J., 2011. Assessment of resistance risk in *Spodoptera exigua* (Hübner) (Lepidoptera: Noctuidae) to chlorantraniliprole. Pest Manag. Sci. 67(11), 1468-1472.

Lara-Juache, H.R.; Ávila-Hernández, J.G.; Rodríguez-Durán, L.V.; Michel, M.R.; Wong-Paz, J.E.; Muñiz-Márquez, D.B.; Veana, F.; Aguilar-Zárate, M.; Ascacio-Valdés, J.A.; Aguilar-Zárate, P., 2021. Characterization of a Biofilm Bioreactor Designed for the Single-Step Production of Aerial Conidia and Oosporein by *Beauveria bassiana* PQ2. J. Fungi, 7, 582.

Llimós Turet, J., Mauricio Martínez, O., Martí, E., Corchado-Lopo, C., Llenas, L., Gea, T., Ponsà, S., 2020. Brewer's spent grain biotransformation to produce lignocellulolytic enzymes and polyhydroxyalkanoates in a two-stage valorization scheme. Biomass conv. Bioref. https://doi.org/10.1007/s13399-020-00918-4

Liu, Y., Wang, Y., Chen, H-Z., 2018. Two steps gas double-dynamic fermentation enhances growth and spore production of *Conithyrium minitans*. Bioresource Technol. 262, 235-241.

Lopes Perez, C., Perpétua Casciatori, F., Cláudio Thoméo, C., 2019. Strategies for scaling-up packed bed bioreactors for solid-state fermentation: The case of cellulolytic enzymes production by a thermophilic fungus. Chem. Eng. J. 361, 1142-1151.

Lopez-Perez, M., Rodriguez-Gomez, D., Loera, O., 2015. Production of conidia of *Beauveria bassiana* in solid-state culture: current status and future perspectives. Crit. Rev. Biotechnol. 35(3), 334–341.

Lopez-Ramirez, N., Volke-Sepulveda, T., Gaime-Perraud, I., Saucedo-Castañeda, G., Favela-Torres, T., 2018. Effect of stirring on growth and cellulolytic enzymes production by *Trichoderma harzianum* in a novel bench-scale solid-state fermentation bioreactor. Biores. Technol. 265, 291-298.

Maistrou, S., Natsopoulou, M.E., Jensen, A.B., Meyling, N.V., 2020. Virulence traits within a community of the fungal entomopathogen Beauveria: Associations with abundance and distribution. Fungal Ecology, 48, 100992. Manpreet, S., Sawraj, S., Sachin, D., Pankaj, S., Banerjee, U.S., 2005. Influence of process parameters on the production of metabolites in solid-state fermentation. Malalays. J. Microbiol. 1, 1–9.

Mar, T.T., Lumyong, S., 2012. Conidial production of entomopathogenic fungi in solid-state fermentation. KKU Research Journal, 17, 762–768.

Marín, M., 2018. Del residuo al producto: recuperación de enzimas hidrolíticas producidas mediante fermentación en estado sólido y estrategias de residuo cero. PhD thesis. Universitat Autònoma de Barcelona.

Marrone, P.G., 2019. Pesticidal natural products – status and future potential. Pest Manag. Sci. 75, 2325- 2340.

Martínez, O., 2018. Fruit-like and rose-like aroma production via Solid-State Fermentation of sugarcane bagasse: process optimization and production strategies. PhD thesis. Universitat Autònoma de Barcelona.

Martínez-Medina, A., Fernández, I., Pescador, L., Romero-Puertas, M.C., Pozo, M.J., 2019. *Trichoderma harzianum* triggers an early and transient burst of nitric oxide and the upregulation of PHYTOGB1 in tomato roots. Plant Signaling & Behaviour. 14, 1640564.

Markets and markets, biopesticide market research report, 2020. Biopesticides Market by Type (Bioinsecticides, Biofungicides, Bionematicides, and Bioherbicides), Source (Microbials, Biochemicals, and Beneficial Insects), Mode of Application, Formulation, Crop Application, and Region - Global Forecast to 2025. Link: https://www.marketsandmarkets.com/Market-Reports/biopesticides-267.html.

Mascarin, G.M., Jaronski, S.T., 2016. The production and uses of *Beauveria bassiana* as a microbial insecticide. World J. Microbiol. Biotechnol. 32, 1–26.

Mascarin, G.M., Lopes, R.B., Delalibera Jr. I., Fernandes, E.K.K., Luz, C., Faria, M., 2019. Current status and perspectives of fungal entomopathogens used for microbial control of arthropod pests in Brazil. J. Invertebr. Pathol. 165, 46-53.

Mejías, L., 2020. A step towards biowaste digestate valorization: process development for Bt-based biopesticides production through SSF and performance at demonstration scale. PhD thesis. Universitat Autònoma de Barcelona.

Membrillo, I., Sànchez, C., Meneses, M., Favela, E., Loera, O., 2011. Particle geometry affects differentially substrate composition and enzyme profiles by *Pleurotus ostreatus* growing on sugar cane bagasse. Bioresource Technol. 102(2), 1581–1586.

Méndez-González, F., Loera, O., Favela-Torres, E., 2017. Conidia production of *Metarhizium anisopliae* in bags and packed column bioreactors. Current Biotechnol. 5, 1–5.

Méndez-González, F., Loera-Corral, O., Saucedo-Castañeda, G., Favela-Torres, E., 2018. Bioreactors for the production of biological control agents produced by solid-state fermentation. In: Pandey, A., Larroche, C., Soccol, R (eds.). Current developments in biotechnology and bioengineering: current advances in solid-state fermentation., pp. 109-121. ElSevier.

Méndez-González, F., Loera, O., Saucedo-Castañeda, G., Favela-Torres, E., 2020. Forced aeration promotes high production and productivity of infective conidia from *Metarhizium robertsii* in solid-state fermentation. Biochem. Eng. J. 156. 107492.

Meyling, N.V., Eilenberg, J., 2007. Ecology of the entomopathogenic fungi *Beauveria bassiana* and *Metarhizium anisopliae* in temperate agroecosystems: Potential for conservation biological control. Biol. Control. 43(2), 145–155.

Miller G.L. (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal. Chem. 31:426–428.

Mishra, J., Tewari, S., Singh, S., Arora, N. K., 2015. Biopesticides: where we stand? In: Arora, N. (ed) Plant Microbes Symbiosis: Applied Facets. Springer, New Delhi, pp. 37-75.

Mishra, S., Kumar, P., Malik, A., 2016. Suitability of agricultural by-products as production medium for spore production by *Beauveria bassiana* HQ917687. Int. J. Recycl. Org. Waste Agric. 5(2), 179–184.

Mishra, N., Sundari, S.K., 2016. Designing low cost SSF strategy for mass production of bioinoculant *Trichoderma harzianum* KSNM with longer shelf life. Asian J. Microb. Biot. Environ. Sci. 18(2), 447-458.

Mnif, I., Ghribi, D., 2015. Potential of bacterial derived biopesticides in pest management. Crop Prot. 77, 52–64.

Moore, D., Douro-Kpindou, O.K., Jenkins, N.E., Lomer, C.J., 1996. Effects of moisture content and temperature on storage of *Metarhizium flavoviride* conidia. Biocontrol Sci. Techn. 6(1), 51–62.

Moslim, R., Hamid, N.H., Wahid, M.B., 2005. Mass production of *Metarhizium anisopliae* using solid-state fermentation and wet harvesting methods. Proceedings of the PIPOC 2005 International Palm Oil Congress (Agriculture, Biotechnology and Sustainability), 928–942.

Muñiz-Paredes, F., Garza-López, P.M., Viniegra-González, G., Loera, O., 2016. Comparison between superficial and solid-state cultures of *Isaria fumosorosea*: conidial yields, quality and sensitivity to oxidant conditions. World J. Microb.Biot. 32, 111.

Muñiz-Paredes, F., Miranda-Hernández, F., Loera, O., 2017. Production of conidia by entomopathogenic fungi: from inoculants to final quality tests. World J. Microb. Biot. 33,1–9.

Muñoz, G.A., Agosin, E., Cotoras, M., Martin, R.S., Volpe, D., 1995. Comparison of aerial and submerged spore properties for *Trichoderma harzianum*. FEMS Microbiol. Lett. 125(1), 63–69.

Nandakumar, R., Bollich, P.A., Shahjahan, A.K.M., Groth, D.E., Rush, M.C., 2008. Evidence for the soil borne nature of the rice sheath rot and panicle blight pathogen, *Burkholderia gladioli*. Can. J. Plant Pathol. 30, 148-154.

Nigam, P., Pandey, A., 2009. Solid-state fermentation technology for bioconversion of biomass and agricultural residues. In: Nigam, P., Pandey, A (eds.). Biotechnology for agro- industrial residues utilisation, pp. 197–221. Rotterdam: Springer.

Novy, V., Longus, K., Nidetzky, B., 2015. From wheat straw to bioethanol: Integrative analysis of a separate hydrolysis and co-fermentation process with implemented enzyme production. Biotechnol. Biofuels. 8,1–12.

Nuñez-Gaona, O., Saucedo-Castañeda, G., Alatorre-Rosas, R., Loera, O., 2010. Effect of moisture content and inoculum on the growth and conidia production by *Beauveria bassiana* on wheat bran. Braz. Arch. Biol.Techn. 53(4), 771–777.

Omwango, E.O., Nyaga, E., Njagi, M., Orinda, G.O., Wanjau, R.N., 2013. Nutrient enrichment of pineapple waste using *Aspergillus niger* and *Trichoderma viride* by solid state fermentation. Afr. J. Biotechnol. 12, 6193–6196.

Oreste, M., Bubici, G., Poliseno, M., Triggiani, O., Trasco, E., 2012. Pathogenicity of *Beauveria bassiana* (Bals.-Criv.) Vuill.and *Metarhizium anisopliae* (Metchn.) Sorokin against *Galleria melonella* L. and *Tenebrio molitor* L. in laboratory assays. J. Zool. 95, 43-48.

Ortiz, G.E., Guitart, M.E., Cavalitto, S.F., Albertó, E.O., Fernández-Lahore, M., Blasco, M., 2015. Characterization, optimization, and scale-up of cellulases production by *trichoderma reesei* cbs 836.91 in solid-state fermentation using agro-industrial products. Bioprocess Biosyst. Eng. 38, 2117–2128.

Padmavathi, J., Devi, K.U., Rao, C.U.M., 2003. The optimum and tolerance pH range is correlated to colonial morphology in isolates of the entomopathogenic fungus *Beauveria bassiana* – a potential biopesticide. World J. Microb. Biot. 19, 469-477.

Palomas, G., 2021. Producción de quitinasa por fermentación en estado sólido utilizando *Trichoderma harzianum* en reactor de bandejas. Master thesis. Universitat Autònoma de Barcelona.

Pandey, A., 1992. Recent Process developments in solid-state fermentation. Process Biochem. 27(2), 109–117.

Pandey, A., 2003. Current developments in solid-state fermentation. Biochem. Eng. J. 81, 146-161.

Pandey, A., Selvakumar, P., Soccol, R., Nigam, P., 1999. Solid-state fermentation for the production of industrial enzymes. Curr. Sci. 77, 149–162.

Pandey, A., Larroche, C., Soccol, C.R., 2008. General considerations about solid-state fermentation processes, in: Pandey, A., Soccol, C.R., Larroche, C. (Eds.), Current developments in solid-state fermentation, Springer, Asiatech Publishers, New Delhi, pp. 26-48.

Papagianni, M., 2004. Fungal morphology and metabolite production in submerged mycelial processes. Biotechnol. Adv. 22(3), 189–259.

Papavizas, G.C., 1985. *Trichoderma* and *Glicoladium*: biology, ecology and potential for biocontrol. Annu. Rev. Phytopathol. 23(1), 23–54.

Passamani, F.R.F., Hernandes, T., Lopes, N.A., Bastos, S.C., Santiago, W.D., Cardoso, M.G., Batista, L.R., 2014. Effect of temperature, water activity, and pH on growth and production of ochratoxin A by *Aspergillus niger* and *Aspergillus carbonarius* from Brazilian grapes. J. Food Protect. 77(11), 1947-1952.

Pham, T.A., Kim, J.J., Kim, K., 2010. Optimization of solid-state fermentation for improved conidia production of *Beauveria bassiana* as a mycoinsecticide. Mycobiology. 38(2), 137.

Philips, T.W.; Throne, J.E., 2010. Biorational approaches to managing stored-product insect. Annu. Rev. Entomol. 55, 375–397.

Phonphuak, N and Chindaprasirt, P., 2015. 6-Types of wastes, properties and durability of pore-forming waste-based fired masonry bricks. In: Pacheco-Torgal, F., Lourenço, P., Labrincha, J., Chindaprasirt, P., Kumar, S (eds). Eco-efficient masonry bricks and blocks. Design, propeties and durability., pp. 103-127. ElSevier.

Pode, R., 2016. Potential applications of rice husk ash waste from rice husk biomass power plant. Renew. Sustain. Energy Rev. 53, 1468–1485.

Posada-Flórez, F.J., 2008. Production of *Beauveria bassiana* fungal spores on rice to control the coffee berry borer, *Hypothenemus hampei*, in Colombia. J. Insect Sci. 8(41), 1–13.

Pretty, J., Bharucha, Z.P., 2015. Integrated pest management for suitable intensification of agriculture in Asia and Africa. Insects. 6(1), 152-182.

Puyuelo, B., Gea, T., Sánchez, A., 2010. A new control strategy for the composting process based on the oxygen uptake rate. Chem. Eng. J. 165, 161-169.

Puyuelo, B., Ponsà, S., Gea, T., Sánchez, A., 2011. Determining C/N ratios for typical organic wastes using biodegradable fractions. Chemosphere. 85(4), 653–659.

Raimbault, M., 1998. General and microbiological aspects of solid substrate fermentation. Electron. J. Biotechno. 1,1–15.

Ramachandran, S., Larroche, C., Pandey, A., 2008. Production of spores, in: Pandey, A., Soccol, C.R., Larroche, C. (Eds.), Current developments in solid-state fermentation. Springer, Asiatech Publishers, New Delhi, pp. 230-252.

Rehner, S.A., Minnis, A.M., Sung, G-H, Luangsa-ard, J.J., Devotto, L., Humber, R.A., 2011. Phylogeny and systematics of the anamorphic, entomopathogenic genus *Beauveria*. Mycologia 103:1055–1073.

Reiss, J., 1973 Toxicity of molds to the larvae of *Tenebrio molitor*. J. Invertebr. Pathol. 21, 112–113.

Richard, T.L., Veeken, A.H.M., de Wilde, V., Hamelers, H.V.M., 2004. Air-filled porosity and permeability relationships during solid-state fermentation. Biotechnol. Progr. 20, 1372-1381.

Rodríguez-Gómez, D., Loera, O., Saucedo-Castañeda, G., Viniegra-González, G., 2009. Substrate influence on physiology and virulence of *Beauveria bassiana* acting on larvae and adults of *Tenebrio molitor*. World J. Microb. Biot. 25(3), 513–518.

Ross, C., Opel, V., Scherlach, K., Hertweck, C., 2014. Biosynthesis of antifungal and antibacterial polyketides by *Burkholderia gladioli* in coculture with *Rhizopus microsporus*. Mycoses. 57 (supplement 3), 48-55.

Ruggieri, L., Gea, T., Artola, A., Sánchez, A., 2009. Air filled porosity measurements by air pycnometry in the composting process: A review and a correlation analysis. Bioresource Technol. 100, 2655-2666.

Russ, W., Schnappinger., 2007. Waste related to the food industry: a challenge in material loops, in: Oreopoulou, V., Russ, W. (Eds.), Utilization of by-products and treatment of waste in the food industry. Springer, pp 1-14.

Safavi, S.A., Shah, F.A., Pakdel, A.K., Reza Rasoulian, G., Bandani, A.R., Butt, T.M, 2007. Effect of nutrition on growth and virulence of the entomopathogenic fungus *Beauveria bassiana*. FEMS Microbiol. Lett. 270(1), 116–123.

Sánchez, A., 2007. A kinetic analysis of solid waste composting at optimal conditions. Waste Manag. 27, 854-855.

Sánchez, C., 2009. Lignocellulosic residues: Biodegradation and bioconversion by fungi. Biotechnol. Adv. 27(2), 185–194.

Sánchez-Corzo, L.D., Álvarez-Gutiérrez, P.E., Meca-Gordillo, R., Villalobos-Maldonado, J.J., Enciso-Pinto, S., Enciso-Sáez, S., 2021. Lignocellulolytic enzyme production from wood rot fungi collected in Chiapas, Mexico, and their growth on lignocellulosic material. J. Fungi. 7, 450.

Sandhya, C., Krishna, L., Madhavan, K., Binod, P., Szakacs, G., Pandey, A., 2004. Extracellular chitinase production by Trichoderma harzianum in submerged fermentation. J. Basic Microb. 44, 49-58.

Santa, H.S.D., Santa, O.R.D., Brand, D., Vandenberghe, L.P.D.S., Soccol, C.R., 2005. Spore production of *Beauveria bassiana* from agro-industrial residues. Braz. Arch. Biol. Technol. 48, 51–60.

Schrank, A., Vainstein, M.H., 2010. *Metarhizium anisopliae* enzymes and toxins. Toxicon: Official Journal of the International Society on Toxicology. 56(7), 1267–1274.

Schuster, A., Schmoll, M., 2010. Biology and biotechnology of *Trichoderma*. Appl. Microbiol. Biot. 87(3), 787–799.

Scott, T.A, Jr; Melvin, E.H., 1953. Determination of dextran with anthrone. Analytical Chemistry. 25, 11, 1656 – 1661.

Seid, A.M., Fredensborg, B.L., Steinwender, B.M., Meyling, N.V., 2019. Temperature-dependent germination, growth and co-infection of *Beauveria* spp. isolates from different climatic regions. Biocontrol Sci. Tech. 29(5), 411-426.

Shapiro-Ilan, D.I., Fuxa, J.R., Lacey, L.A., Onstad, D.W., Kaya, H.K., 2005. Definitions of pathogenicity and virulence in invertebrate pathology. J. Invertebr. Pathol. 88, 1-7.

Sharma, S., Gupta, R.B.L., Yadava, C.P.S., 2002. Selection of a suitable medium for mass multiplication of entomofungal pathogens. Indian J. Entomol. 14, 255–261.

Sharma, P., Sharma, M., Raja, M., Shanmugam, V. 2014. Status of *Trichoderma* research in India: A review. Indian Phytopathology. 67, 1-19.

Simione, F.P., 2009. Thermo Scientific nalgene and nunc cryopreservation guide. Thermo Fisher Scientific Inc.

Smalling, K.L., Kuivila, K.M., Orlando, J.L., Phillips, B.M., Anderson, B.S., Siegler, K., Hunt, J.W., Hamilton, M., 2013. Environmental fate of fungicides and other current-use pesticides in a central Californian estuary. Mar. Pollut. Bull. 73(1), 144 - 153.

Smith, C., Edgington, S., 2011. Germination at different water activities of similarly aged *Metarhizium* conidia harvested from ageing cultures. J. Stored Prod. Res. 47(3), 157–160.

Soccol, C.R., Costa, E.S.F. da, Letti, L.A.J., Karp, S.G., Woiciechowski, A.L., Vandenberghe, L.P. de S., 2017. Recent developments and innovations in solid state fermentation. Biotechnol. Res. Innov. 1, 52–71.

Souza, R.K.F., Azevedo, R.F.F., Lobo, A.O., Rangel, D.E.N., 2014. Conidial water affinity is an important characteristic for thermotolerance in entomopathogenic fungi. Biocontrol Sci. Technol. 24(4), 448–461.

Srivastava, M., Kumar, V., Shahid, M., Pandey, S., Singh, A., 2016. *Trichoderma* - a potential and effective bio fungicide and alternative source against notable phytopathogens: A review. Afr. J. Agric. Res. 11, 310–316.

Statista. Value of the global market for crop protection 2008 to 2018. Consulted: April 2021. Available at: https://www.statista.com/statistics/272493/revenue-of-the-plant-protection-market-worldwide-since-2007/

Stenersen, J., 2004. Chemical pesticides mode of action and toxicology. Boca Raton, FL: CRC Press.

Stenmark, A., Jensen, C., Quested, T., Moates, G., 2016. Fusions: estimates of European food waste levels. European Comission, Coordination and Support Action. Available at: http://eu-fusions.org/phocadownload/Publications/Estimates%20of%20European%20food%20waste%20levels.pdf

Stoyanova, M., Pavlina, I., Moncheva, P., Bogatzevska, N., 2007. Biodiversity and incidence of *Burkholderia* species. Biotechnology & Biotechnological Equipment. 21, 306-310.

Streit, E., Schatzmayr, G., Tassis, P., Tzika, E., Marin, D., Taranu, I., Tabus, C., Nicolau, A., Aprodu, I., Puel, O., Oswald, I, P., 2012. Current situation of micotoxin contamination and co-ocurrence in animal feed – focus on Europe. Toxins 4, 788-809.

Swiergiel, W., Meyling, N.V., Porcel, M., Rämert, B., 2016. Soil application of *Beauveria bassiana* GHA against apple sawfly (Hoplocampa testudinea Klug; Hymenoptera: Tenthredinidea): field mortality and fungal persistence. Insect Sci. 23, 854-868.

Tall, S., Meyling, N.V.M., 2018. Probiotics for plants? Growth promotion by the entomopathogenic fungus *Beauveria bassiana* depends on nutrient availability. Microb. Ecol. 76, 1002-1008.

Tarocco, F., Lecuona, R.E., Couto, A.S., Arcas, J. A., 2005. Optimization of erythritol and glycerol accumulation in conidia of *Beauveria bassiana* by solid-state fermentation, using response surface methodology. Appl. Microbiol. Biot. 68(4), 481–488.

Thakore, I., 2006. The biopesticide market for global agricultural use. Genet. Eng. Biotechnol. 2, 194-208.

The US Department of Agriculture and The US Composting Council. 2001. Test methods for the examination of composting and compost. Houston: Edaphos International.

Thomas, L., Larroche, C., Pandey, A., 2013. Current developments in solid-state fermentation. Biochem. Eng. J. 81, 146–161.

Thomas, M.B., Jenkins, N.E., 1997. Effects of temperature on growth of *Metarhizium flavoviride* and virulence to the variegated grasshopper, *Zonocerus variegatus*. Mycol. Res. 101(12), 1469–1474.

Tronsmo, A., Dennis, C., 1978. Effect of temperature on antagonistic properties of Trichoderma species. T. Brit. Mycol. Soc. 71(3), 469–474.

Ugine, T.A., 2011. The effect of temperature and exposure to *Beauveria bassiana* on tarnished plant bug *Lygus lineolaris* (Heteroptera: Miridae) population dynamics, and the broader implications of treating insects with entomopathogenic fungi over a range of temperatures. Biol. Control. 59(3), 373–383.

Velasco-Alvarez, N., Gutiérrez-Rojas, M., González, I., 2017. Alterations in *Aspergillus brasiliensis* ATCC 9642 membranes associated to metabolism modifications during application of low-intensity electric current, Bioelectrochemistry. 118, 1–7.

Verma, M., Brar, S.K., Tyagi, R.D., Surampalli, R.Y., Valéro, J.R. (2007). Antagonistic fungi, *Trichoderma* spp.: Panoply of biological control. Biochem. Eng. J. 37(1), 1–20.

Vestergaard, S., Butt, T.M., Bresciani, J., Gillespie, A.T., Eilenberg, J., 1999. Light and electron microscopy studies of the infection of the western flower thrips *Frankliniella occidentalis* (Thysanoptera: Thripidae) by the entomopathogenic fungus *Metarhizium anisopliae*. J. Invertebr. Pathol. 73, 25–33.

Wilson, S.M. and Hulla, W.D., 2019. Multivariate approaches to understanding aphasia and its neural substrates. Curr. Neurol. Neurosci. Rep 19:53.

Wraight, S.P., Ramos, M.E., Avery P.B., Jaronski, S.T., Vandenberg, J.D., 2010. Comparative virulence of *Beauveria bassiana* isolates against lepidopteran pests of vegetable crops. J. Inv. Pathol. 103 (3), 186-199.

Wraight, S.P., Ugine, T.A., Ramos, M.E., Sanderson, J.P., 2016. Efficacy of spray applications of entomopathogenic fungi against western flower thrips infesting greenhouse impatiens under variable moisture conditions. Biolog. Contr. 97, 31-47.

Xie, L., Chen, H.M., Yang, J.B., 2013. Conidia production by *Beauveria bassiana* on rice in solid-state fermentation using tray bioreactor. Adv. Mat. Res. 610–613, 3478–3482.

Xie, L., Han, J.H., Kim, J.J., Lee, S.Y., 2016. Effects of culture conditions on conidial production of the sweet potato whitefly pathogenic fungus *Isaria javanica*. Mycoscience. 57, 64–70.

Yang, H., Li, J., Sun, J., Liu, L., Du, G., Chen, J., 2010. Statistical model based optimization of spore production by solid-state culture of *Beauveria bassiana*. Biocontrol Sci. Techn. 20(10), 1087–1095.

Yang, Y-T., Lee, M.R., Lee, S.J., Kin, S., Nai, Y-S., Kim, J.S., 2018. *Tenebrio molitor* Gram-negative-binding protein 3 (*TmGNBP3*) is essential for inducing downstream antifungal *Tenecin 1* gene expression against infection with *Beauveria bassiana* JEF-007. Insect Sci. 25, 969-977.

Yazid, N.A., Barrena, R., Komilis, D., Sánchez, A., 2017. Solid-state fermentation as a novel paradigm for organic waste valorization: A review. Sustainability. 9,1–28.

Ye, S. D., Ying, S. H., Chen, C., Feng, M. G., 2006. New solid-state fermentation chamber for bulk production of aerial conidia of fungal biocontrol agents on rice. Biotechnol. Lett. 28(11), 799–804.

Zhang, J.D., Yang, Q., 2015. Optimization of solid-state fermentation conditions for *Trichoderma harzianum* using an orthogonal test. Genet. Mol. Res. 14(1), 1771–1781.

Zhang, Y., Liu, J., Zhou, Y., Ge, Y., 2014. Spore Production of *Clonostachys rosea* in a New Solid-state Fermentation Reactor. Appl. Biochem. Biotech. 174(8), 2951–2959.

Zimmermann, G., 2007. Review on safety of the entomopathogenic fungi *Beauveria bassiana* and *Beauveria brongniartii*. Biocontrol Sci. Technol. 17: 553–596.