PPS-jaarrapportage 2019
De PPS-en die van start zijn gegaan onder aansturing van de topsectoren dienen jaarlijks te rapporteren over de inhoudelijke en financiële voortgang. Voor de inhoudelijke voortgang dient dit format gebruikt te worden. Voor PPS-en die in 2019 zijn afgerond is een apart format "PPS-eindrapportage" beschikbaar.
De jaarrapportages worden integraal gepubliceerd op de website het TKI's. Zorg er svp voor dat er geen vertrouwelijke zaken in staan.

De PPS-jaarrapportages dienen voor 1 maart 2020 te worden aangeleverd bij finance@tki-bbe.nl.

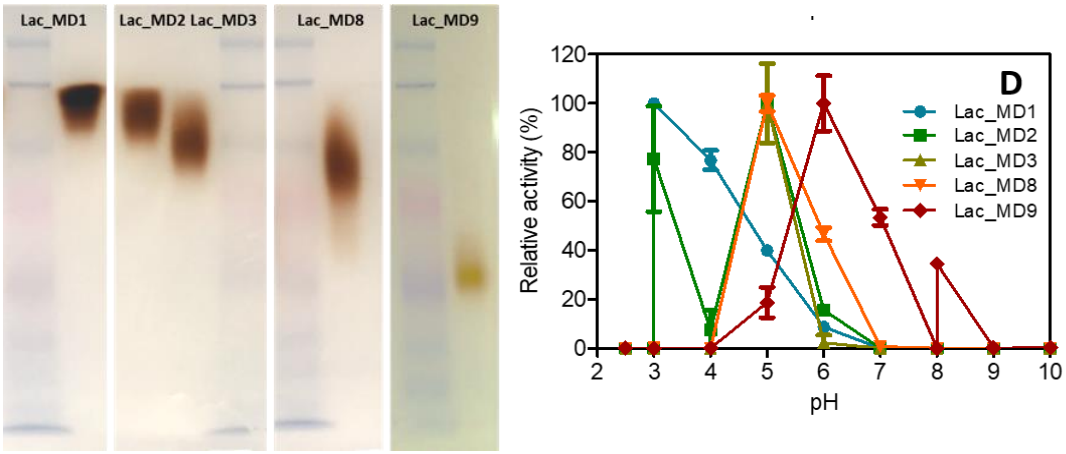
Algemene gegevens	
PPS-nummer	**TKI-BBE/1607**
Titel	**BIOCOM**
Roadmap	**Sterktes in Innovatie**
Uitvoerende kennisinstelling(en)	**TU Delft/VU**
Projectleider onderzoek (naam + emailadres)	**Dr. Tjalf de Boer,** tjalf.de.boer@microlifesolutions.nl
Penvoerder (namens private partijen)	**Microlife Solutions B.V.**
Contactpersoon overheid (indien relevant)	**TKI-BBE**
Adres projectwebsite	
Startdatum	**01-01-2017**
Einddatum	**31-12-2020**

Goedkeuring penvoerder / consortium De jaarrapportage dient te worden besproken met de penvoerder/het consortium. TKI BBE neemt graag kennis van evt. opmerkingen over de jaarrapportage.	
De penvoerder heeft namens het consortium de jaarrapportage	X goedgekeurd □ niet goedgekeurd
Evt. opmerkingen over de jaarrapportage:	De jaarrapportage 2019 omvat voortgang van de partners MLS, BDS en VU; terwijl in 2019 TU Delft, DSM en BPF hebben gewerkt aan het ontwerpen van een gewijzigd projectplan voor hun deel (milestone 8), dat in het najaar van 2019 is ingediend en pas in januari 2020 is gestart. Alhoewel deze studies succesvol zijn gestart in januari-februari 2020, gooit de corona crisis de planning danig in de war. Door de lock down van, met name, universitaire laboratoria in de periode van maart-juni zullen naar verwachting de uitvoering van pilot experimenten ook opschuiven met 4 maanden. **Om een en ander te kunnen afronden verzoeken wij derhalve om verlenging van de einddatum van BIOCOM tot 30 april 2021.**

Inhoudelijke samenvatting van het project	
Probleemomschrijving	De huidige 2e generatie BBE maakt primair gebruik van de koolhydraat-component van lignocellulose, dat stevig verpakt zit in lignine vezels van een grote diversiteit aan biorenewable feedstocks. Om deze koolhydraten en de suikers hier efficient uit te kunnen halen worden de feedstocks via een relatief brute wijze uit hun matrix ontsloten via zg steam explosion technieken bij zure of alkalische conditie. In dit voorstel wordt door MLS en VU onderzocht of er een meer efficiente, energie- en milieuvriendelijke biologische manier kan worden gevonden om suikers te ontsluiten uit biomassa, , gebruikmakend van e.g., schimmelenzymen uit e.g., hotsprings die in

	staat zijn om zeer recalcitrante organische moleculen af te breken (contaminanten, natuurlijke recalcitrante stoffen). Ook wordt door BDS aandacht besteed aan de aanwezigheid van natuurlijke bioactieve stoffen, die indien aanwezig, eerst uit biomassa zouden moeten worden gewonnen voordat dit de pretreatment processing in gaat. Verder wordt gekeken naar de safety aspecten van diverse biomassa bronnen. Bij het wijzigingsvoorstel voor het gedeelte uitgevoerd door TU Delft, BPF en DSM is de focus verlegd naar het onderzoeken of CO2 afvang via omzetting naar formic acid kan worden gerealiseerd en dit energetisch nuttig kan worden gebruikt als co-feeding grondstof in de fermentatie reactie van lignocellulose naar chemische bouwstenen.
Doelen van het project	For MLS/VU, the overall aim of the BIOCOM project is to investigate the opportunities of the addition of microbial-based solutions and optimizations to biomass pretreatment and test those against conventional pretreatment and fermentation at pilot scale. In this project we will investigate the possibilities of using microbial enzymes for biomass pretreatment, as well as, for the removal of fermentation inhibitors and determine if enzymatic breakdown of biomass has a positive or negative impact on human health. BDS is investigating the safety aspects of biomass and presence of natural bioactive compounds in biomass and its possibilities to retrieve them from the biomass, prior to its pretreatment and bioconversion. For TU-Delft, BPF and DSM the main aim of the adjusted project for milestone 8 is to investigate the opportunities to reduce both glucose consumption and CO_2 emission by fermentative production of food, feed, fuel, pharmaceuticals and materials, through the capture of CO_2 in the offgas, reduce it to formic acid -using renewable electricity- and co-feed the formic acid with the glucose to the fermentation. Proof of principle for this concept has been delivered in the past for *Saccharomyces cerevisiae* and *Penicillium chrysogenum*. The energetics of the industrially important biotechnological host *Yarrowia lipolytica* with a co-feeding of glucose and formic acid has by our knowledge never been reported. Neither have practical aspects of industrialization of such a process on pilot or larger scale been described in open literature. This subproject aims to investigate both aspects; the former (energetics) at TU Delft and the latter (industrialization) at BPF.

Resultaten	
Beoogde resultaten 2019	MLS and VU: In 2018 we reported on the MICROGLO® fermentation inhibitor analysis and subsequent breakdown by extracellular white-rot fungal enzymes. These enzymes were able to break down 71% of the tested fermentation inhibitor and will we expect that, the majority of additional fermentation inhibiting compounds will also be degraded. Previously, we reported on the sequencing of the Rigidoporus FMD21 genome and the heterologous overexpression of seven laccase genes found in this genome. Although we were able to express the laccase genes into active enzymes the overall yield in the expression host Pichia pastoris was low. Especially compared to the laccase yield shown by the native fungus FMD21. We therefore decided to focus more on native laccase enzymes expressed by the original fungus. Using different extraction and isolation techniques we expect to isolate different laccase genes with different activities and general properties. Work on BIOCOM milestone 2D showed that while FMD21 extra cellular enzymes can breakdown lignin model compounds it remained unclear what its preference for cellulose or lignin would be when tested on plant biomass. In 2019 we will test this using hemp

	straw and we will also test alternative white rot fungi from our collection. BDS: The main aim of BDS in 2019 is the valuation of biomass by bioactivity screening using human HTP screening system by BDS focusing on: a) Detection and identification of valuable natural bioactive compounds in biomass prior to its cascaded fractionation to capture all value available in biomass b) safety evaluation of biomass and biobased building blocks thereof using its HTP CALUX screening system. TU-Delft, BPF and DSM did not perform experimental work in 2019 on the adjusted work package for milestone 8 (see annex). They started their work at beginning of 2020
Behaalde resultaten 2019	**MLS and VU** **Laccase isoform characterization** Previous experiments using heterologous expressed laccase gene from the white-rot fungus FMD21 using a Pichia pastoris expression system showed active enzyme but rather low yields per liter of growth medium. When grown on optimal medium, FMD21 secretes large amount of laccase but it is not clear if all laccase genes (FMD21 has at least 7) are expressed and what the ratios of expression are between the different laccase genes. Using protein chromatography isolation and separation techniques we isolated 5 different laccase isoforms. Figure 1 left shows enzyme activity gel electrophoresis results on native laccase proteins. All laccase isoforms show activity in the form of a brown color effects where the substrate guaiacol is oxidized. The different isoforms all end up on different places in the gel showing differences in isoelectric point for each protein clearly indicating at least 5 unique laccase proteins. Optimal pH analysis of the 5 isolated isoforms (figure 1, right) showed different pH properties for each laccase enzyme where lac_MD9 was mostly active at elevated pH (5-8) while lac_MD1 and lac_MD2 were active at lower pH (3-5). This isoform isolation was performed at lab scale but will be performed at larger scale in the near future which means that we can investigate per isoform how biomass and fermentation inhibitors are degraded by the different FMD21 laccase isoforms  *Figure 1. Left: gel electrophoresis results of isolated laccase isoforms derived from the FMD21 fungus. Each lane represents a different laccase isoform with different isoelectric properties. Right: pH preference of the different laccase isoforms.* **Biomass solid state fermentation**

Biological pretreatment of biomass can potentially be used to lower the need for chemical or thermal pretreatment thereby saving on both energy and chemicals. We previously showed that white-rot fungi can be used to degrade lignin and could be used to pretreat lignocellulose to open up the lignocellulosic structure prior to the use of other pretreatment techniques. A possible downside of biological pretreatment is that there is a chance that the used microorganisms also consume target compounds such as cellulose. Previously we tested the dioxin degrading white-rot fungus FMD21 for lignin degradation and we found that lignin model compounds were readily degraded by both the fungus and extracellular fungal enzymes which are secreted during the growth phase. In subsequent experiments using live fungus in the solid-state fermentation of hemp straw we found however that mainly carbohydrates such as cellulose were consumed, and lignin was unchanged. Figure 2 shows the concentrations of free glucose that could be obtained from treated hemp straw. The fungus treated samples showed the lowest concentration which indicated that part of the cellulose, from which glucose is obtained, has been consumed by the fungus. Several strategies could be used to prevent this. For example, extracellular enzymes,

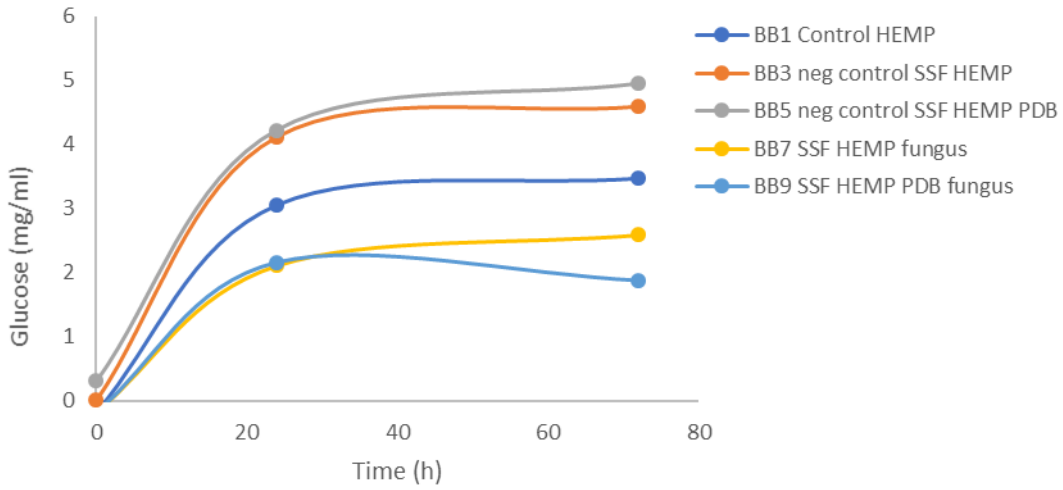


Figure 2. Concentration of glucose derived from several different forms of pretreated hemp straw using incubation with cellulase.

which only depolymerize lignocellulose and not consume it, could be used instead of live fungus. The drawback from using enzymes is loss of activity and that enzymes work optimally in liquid treatment and not very well in solid-state fermentation. Another method could be to use other fungi. Some white-rot fungi only grow on dead wood in late state decay which is depleted of cellulose and only contains lignin. We screened two additional fungi from our library for growth on different polymers and initial experiments look promising. Overall laccase activity of these additional fungi is lower than FMD21 but could still lead to considerable depolymerization of biomass.

Pesticide detoxification using relevant soil borne bacteria (VU)

When biomass is used to produce biofuels or chemical building blocks there is a risk that contaminants in the biomass will end up in the intermediates or even the final end products. For example, pesticides used during the biomass growth phase could potentially end up in the biomass and be hazardous for both microorganisms used for fermentation as well as for human health. Bacteria isolated from soils which are contaminated with pesticides could have the potential for degradation of pesticides. To test this soil was sampled in an area contaminated with the two chemical compounds that make up the rainbow herbicide Agent Orange (2,4,5-T and 2,4-D). The bacterial community contained in this soil was subjected to bacterial enrichment experiments which means that the soil bacteria were grown on either or both chemical compound as a single carbon source. Bacteria that are able to grow on either or both chemicals contain enzymes that can degrade the herbicides and were subsequently isolated from the community. From the enriched culture 4 bacterial isolates were selected for further experiments (see figure **x**). All 4 bacteria were able to grow on the two chemicals (figure 3A) when used as a single carbon source and were able to degrade the chemicals fully within 10 days (figure 3B & 3C). The genomes for all

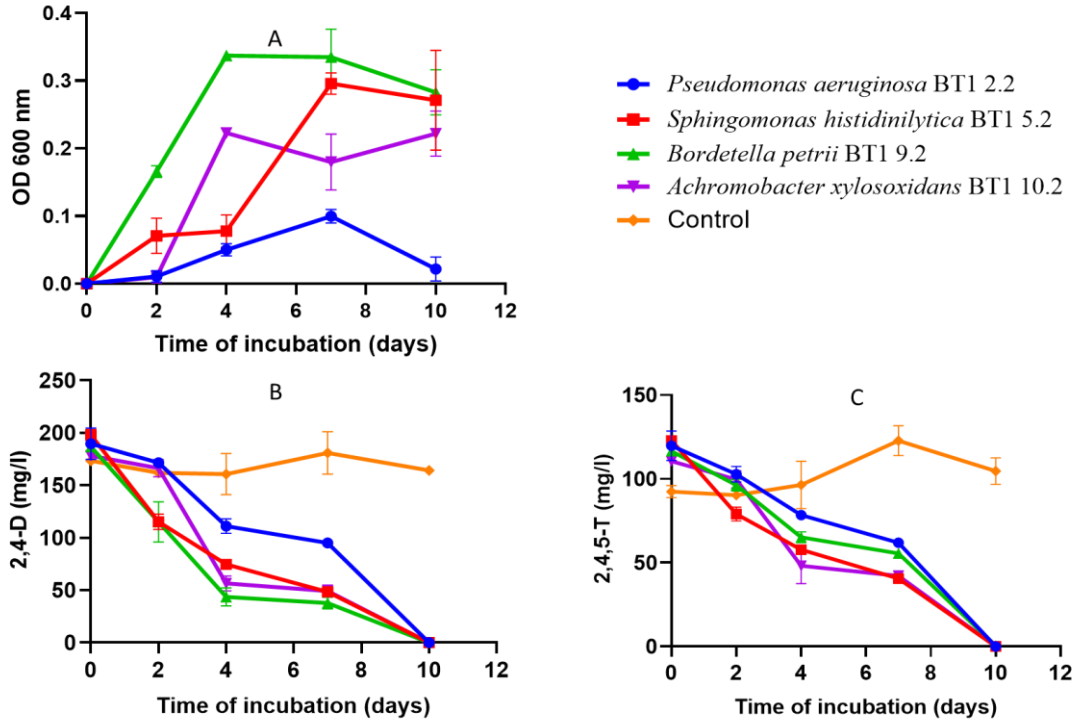


Figure 3, upper graph: Bacterial growth measured as optical density of the 4 bacterial isolates derived from the enriched soil bacterial culture. Lower graphs: pesticide breakdown over time from the 4 isolates with 2,4-D on the right and 2,4,5-T on the left.

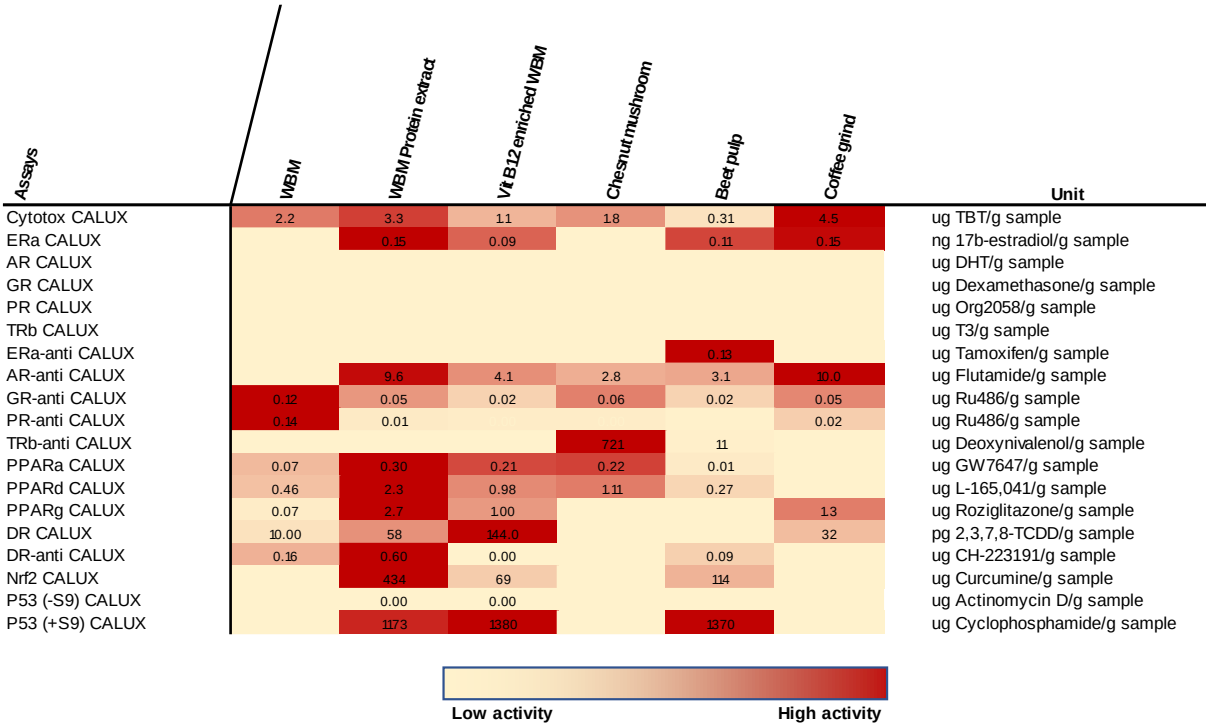
4 bacteria were sequenced and analyzed for relevant degradation pathways. Bordetella petri, one of 4 selected isolates, contained a full complement of known 2,4-T and 2,4,5-T

degradation enzymes where the other 3 bacteria contained several combinations of the different enzymes. This also explains why B. petri was the fastest grower on the chemicals. Further research will be performed on this bacterium to determine degradation of other contaminants and its usefulness in biological remediation.

BDS:

Following the previous selection and further optimization of Accelerated Solvent Extract (ASE) as suitable extraction method for a wide range of biomass products (see report 2018), additional biomass samples were extracted and analyzed on a wide panel of CALUX bioassays to assess bioactivity present in these extracts. In figure 1, a heat-map of analysis results of a selected set of biomass samples is shown. Results indicated that the different types of biomass extracts show different types of bioactivity with mushroom extracts being particular active with respect to PPAR-activity and antagonistic endocrine activity.

Figure 1 Quantified wide panel CALUX analysis results of selected biomass sample extracts (White button mushroom varieties; chestnut mushroom, beet pulp; coffee grind).



For toxicological evaluation of biomass products, safety thresholds have to be determined for each of the selected effect-based bioassays. To establish such safety limits (or effect-based trigger values; EBTs) several approaches (statistical (Escher et al (2015)), theoretical (Brand et al (2013)) or practical (Besselink et al. (2017)) can be taken. Because of the complex nature of mushroom samples, the lack of information on bioactivity of compounds present in mushrooms (if known at all) and the limited availability of regulated chemical guideline values for mushrooms, the statistical and theoretical approach to derive EBTs for e.g. WBM was not feasible. Furthermore, since also a very large data set of relevant reference samples bioactivities to derive practical EBTs is currently not available, an alternative pragmatic approach to establish EBTs for biomass such as WBM was developed.

An initial evaluation and classification of the analysis results of the wide panel CALUX screening of various mushroom varieties and other biomass samples, based on the derived biomass EBTs, is presented in Figure 2. This approach allows for evaluation of toxicity (or safety) of mushroom varieties and other biomass samples for each specific bioassay (figure 2-left) and overall weighted bioactivity (figure 2-right).

Figure 2 Classification of wide panel CALUX analysis results of mushroom varieties and related biomass samples based on pragmatic derived EBT. Color-coding is applied to indicate the exceedance of the EBTs.

Quantified wide panel CALUX analysis results

Assays	WBM	WBM Protein extract	Vit B12 enriched WBM	Chesnut mushroom	Beet pulp	Coffee grind
Cytotox CALUX	2.2	3.3	1.1	1.8	0.31	4.5
ERa CALUX	LOQ	0.15	0.09	LOQ	0.11	0.15
AR CALUX	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ
GR CALUX	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ
PR CALUX	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ
ERa-anti CALUX	LOQ	LOQ	LOQ	LOQ	0.13	LOQ
AR-anti CALUX	LOQ	10	4	2.84	3.13	10
GR-anti CALUX	0.12	0.05	0.02	0.06	0.02	0.05
PR-anti CALUX	0.14	0.0051	LOQ	LOQ	LOQ	0.022
PPARa CALUX	70	300	210	22	10	LOQ
PPARd CALUX	460	2260	980	1110	270	LOQ
PPARg CALUX	70	2710	1000	LOQ	LOQ	1320
DR CALUX	10.00	58.00	144	LOQ	LOQ	32.00
Nrf2 CALUX	LOQ	434	69	LOQ	114	LOQ
P53 (-S9) CALUX	LOQ	LOQ	LOQ	LOQ	LOQ	LOQ
P53 (+S9) CALUX	LOQ	1173	1380	LOQ	1370	LOQ

Fold exceedence pragmatic derived EBTs

	WBM	WBM Protein extract	Vit B12 enriched WBM	Chesnut mushroom	Beet pulp	Coffee grind
	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
	0.0	3.4	1.5	1.0	1.1	3.6
	6.5	2.4	0.0	3.0	0.0	2.9
	0.0	0.0	0.0	0.0	0.0	0.0
	16	68	48	5.0	2.3	0.0
	3.5	17	7.5	8.5	2.1	0.0
	3.8	149	55	0.0	0.0	73
	0.0	2.6	6.5	0.0	0.0	1.5
	0.0	109	17	0.0	28	0.0
	0.0	0.0	0.0	0.0	0.0	0.0
+	0.0	5.3	6.3	0.0	6.2	0.0
Weighted activity	2.0	24	9.5	1.2	2.7	5.4

Classification individual activities

- analysis result < 1*EBT
- 1*EBT < analysis result < 3*EBT
- 3*EBT < analysis result < 10*EBT
- 10*EBT < analysis result < 100*EBT
- analysis result > 100*EBT

Classification weighted activities

- analysis result < 1
- 1 < analysis result < 3
- 3 < analysis result < 10
- 10 < analysis result < 100
- analysis result > 100

In the heat-map presented in figure 1, the CALUX analysis results from mushroom varieties extracts indicate the presence of compounds showing PPAR-activity which is associated with a.o. maintaining fat balance. Compounds showing high potency to activate PPARs present in mushroom might therefore be valuable natural bioactive compounds that can be used for treatment of e.g. obesitas.

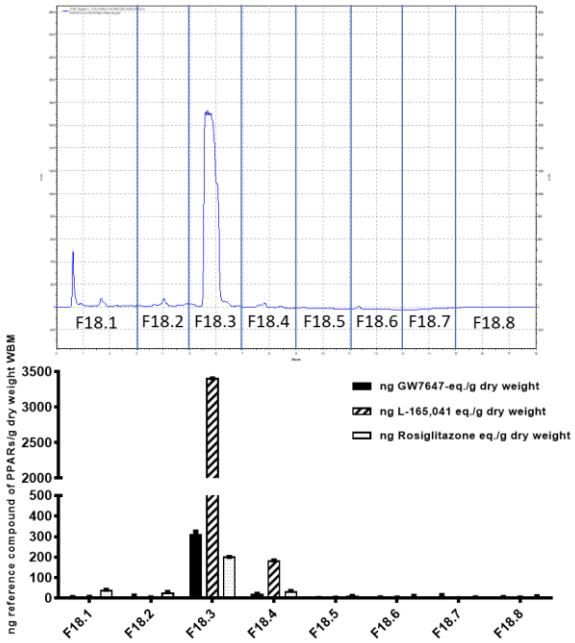


Figure 3 Chromatogram of C18 fractionation (2 min.) of WBM and PPAR CALUX bioactivity response in collected fractions

To identify such compounds, present in WBM extracts and responsible for the observed PPARa, PPARd and PPARg bioactivities, the WBM extract were fractionated using multiple HPLC fractionation steps (biphenyl, Fluor-5 and C18 as stationary phase). During fractionation experiments all fraction were collected and tested for the presence of PPAR CALUX activity. Fractions showing such activity were further fractionated. In figure 3 PPAR CALUX activity in collected fractions, following extensive fractionation is presented. The fraction containing high PPAR CALUX activity was used for identification of compounds present in this fraction using LC/MS-TOF analysis. Results indicate linoleic acid or linoleic acid isoforms as most probable candidate present in the tested fraction as PPAR CALUX activator. Subsequent testing of LC/MS-TOF tested fractions and Linoleic acid confirmed that linoleic acid is an activator of the PPAR CALUX bioassays (see figure 4). However, these studies also show that the observed bioactivity in WBM fractionated extracts cannot be fully assigned to linoleic acid and further CALUX testing of linoleic acid derivatives/isoforms is required.

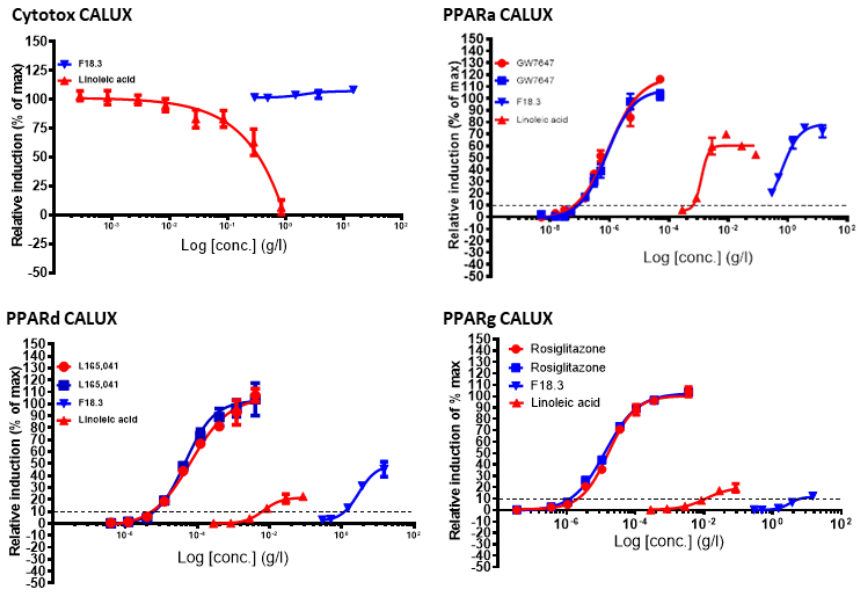


Figure 4 Cytox and PPARa,d,g CALUX analysis results WBM fraction F18s.3

Beoogde resultaten 2020

MLS

In 2020 the focus of the project will shift somewhat, related to the adjusted work package of milestone 8 (see annex to this report). In corporation with the different partners it was decided to shift the second part of the BIOCOM project to the capture of CO2 using formic acid as this will contribute to the development of sustainable fermentation reactions of biomass. Using formic acid in bioreactors however could have consequences for both equipment as well as microorganisms use during fermentation. For example, formic acid could react with fermentation inhibitors present in the feedstock which would alter their properties and could influence how they react with both microorganisms as well is with fermentation inhibiting enzymes.

BDS

In 2020 the focus will be mostly on how to setup a generic safety evaluation for various sources of biomass as feedstock and we expect to deliver a standardized protocol for this.

TU-Delft, BPF and DSM

In 2020 the studies will be focused on the co-feeding of formic acid and glucose to the industrially important biotechnological host *Yarrowia lipolytica*. both at lab. Scale (TU Delft) and at pilot scale (BPF) The expected outcome is:

- Report TU Delft describing the physiology of *Yarrowia lipolytica* grown in chemostat at various glucose/ formic acid feeding ratios and/or dilution rates.
- Report BPF describing fed batch fermentations of *Yarrowia lipolytica* at 10-20L and 300L scale, with feed of glucose with and without formic acid co-feeding. Report also described practical aspects of handling formic acid on pilot and larger scale (corrosion, safety, sterilization, …).

Although these studies were successfully started in January/February of 2020, the corona crisis has blocked the performance of studies at lab scale (TU Delft) in the period March-June for almost 4 months. This will also result in delays of executing the pilot scale studies at BPF and for that reason we request for an extension of the end date of this project until end of April 2021.

Opgeleverde producten in 2019 (geef de titels en/of omschrijvingen van de producten / deliverables of een link naar de producten op de projectwebsite of andere openbare websites)

Wetenschappelijke artikelen:

Characterization of 2,3,7,8-tetrachlorodibenzo-p-dioxin biodegradation by extracellular lignin-modifying enzymes from ligninolytic fungus. Anh T.N. Dao, Sander J. Loenen, Kees Swart, Ha T.C. Dang, Abraham Brouwer and Tjalf E. de Boer. Under review at Chemosphere.

Species and metabolic pathways involved in bioremediation of Vietnamese soil contaminated with Agent Orange. Thi Lan Anh Nguyen, Thi Cam Ha Dang, Jacco Koekkoek, Martin Braster, John R. Parsons, Abraham Brouwer, Tjalf de Boer, Rob J.M. van Spanning. Submitted to Molecular Ecology Resources.

Long Pham Ngoc, Hai yen Man, Harry Besselink, Ha Dang Thi Cam, Abraham Brouwer, Bart van der Burg . (2019). Identification of PPAR-activating compounds in herbal and edible plants and fungi from Vietnam. Industrial Crops and Products. 129, 195-200

Externe rapporten:

Artikelen in vakbladen:

Inleidingen/posters tijdens workshops, congressen en symposia:

SETAC Europe annual meeting 2019. Tjalf E. de Boer, poster presentation

XENOWAC conference 2018. Tjalf E. de Boer, oral presentation

XENOWAC II conference 2018. Harrie Besselink, oral presentation

TV/ Radio / Social Media / Krant:

Overig (Technieken, apparaten, methodes etc.):