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ORIGINAL ARTICLE

Biosynthetic Potential of *Inonotus Obliquus* in Symbiosis With Bacterial Cellulose: Optimisation and Prospects

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Abstract

Background/Aim: *Inonotus obliquus*, commonly known as the Chaga mushroom, is an extensively studied source of bioactive compounds, including polyphenols, triterpenoids and polysaccharides. These metabolites exhibit potent antioxidant, anti-inflammatory and immunomodulatory properties, underscoring *I obliquus* as a valuable resource for advanced biopharmaceuticals and functional therapeutics. Yet, achieving consistent and high-yield biosynthesis of these compounds remains challenging. Aim of this study was to develop a biotechnological approach leveraging co-cultivation with the bacterial cellulose producer *Gluconacetobacter hansenii* to improve both the yield and stability of these bioactive metabolites.

Methods: *I obliquus* was cultivated on solid and liquid-phase substrates, with and without *G hansenii* co-cultivation. Biomass yield, morphological changes and growth dynamics were assessed. Total phenolic content and antioxidant activity were quantified using the Folin-Ciocalteu method and DPPH/ABTS assays. Anti-inflammatory properties were evaluated by measuring IL-6, TNF-α and IL-1β levels in LPS-stimulated THP-1 macrophages *via* ELISA.

Results: Co-cultivation with *G hansenii* resulted in a 1.8-fold increase in fungal biomass yield (30.6 g/L on HS medium) and a 1.3-fold enhancement in phenolic content (85.4 mg GAE/g) compared to monoculture. Extracts from HS medium exhibited the highest antioxidant activity (IC_{50} = 43.2 μg/mL in DPPH and 29.8 μg/mL in ABTS assays) and significantly reduced pro-inflammatory cytokine levels (IL-6 by 47.5 %, TNF-α by 42.8 % and IL-1β by 39.6 %) in LPS-stimulated macrophages. Morphological analysis revealed a denser, more organised fungal network under co-cultivation, suggesting improved metabolic efficiency and bioactive compound synthesis.

Conclusion: Co-cultivation with *G hansenii* provides a scalable and efficient strategy to enhance the bioactive potential of *I obliquus*. This approach significantly improves metabolite yield, antioxidant capacity and anti-inflammatory activity, opening new avenues for the development of standardised therapeutic formulations targeting oxidative stress and immune modulation.

Key words: *Inonotus obliquus*; Cellulose, bacterial; Antioxidants; Activity; Anti-inflammatory effects; Biotechnological cultivation; Phytochemicals.

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Introduction

The rising prevalence of chronic and oncological diseases worldwide underscores the urgent need for safer and more effective therapeutic strategies.[1, 2] While chemotherapy, surgery, radiothera-

py and pharmacology remain standard approaches, their efficacy is often hindered by severe side effects, variability in patient response and sustainability concerns.[3, 4] These factors drive interest in bioactive natural compounds, which offer diverse therapeutic properties and improved safety profiles.

Persistent limitations of conventional therapies have intensified the search for alternative treatment strategies.[5, 6] Although nanotechnology and stem cell therapies show potential, their clinical implementation is limited by high costs, regulatory hurdles and translational challenges. Similarly, bioactive natural compounds exhibit diverse pharmacological properties but face obstacles such as inconsistent composition, lack of standardisation and insufficient validation.[7, 8]

Inonotus obliquus (Chaga mushroom) is a basidiomycetous fungus valued for its pharmacological potential.[9, 10] It predominantly colonises *Betula pendula* Roth across the boreal forests of Europe and North America, with the highest prevalence in taiga and mixed-forest zones. Birch-associated strains exhibit enhanced bioactivity due to their distinct metabolite profile, particularly enriched in betulin-derived triterpenoids and melanin complexes.[11, 12] Traditionally, *I obliquus* has been used to manage gastrointestinal disorders, metabolic dysregulation and malignancies.[13] The fruiting body and sclerotium of *I obliquus* contain a diverse array of bioactive metabolites, including polysaccharides (notably β-glucans), polyphenols and triterpenoids. These compounds are associated with antioxidant, anti-inflammatory, immunomodulatory and antitumor effects.[14] However, translating its therapeutic potential into clinical applications remains challenging due to metabolite variability, instability and difficulties in standardisation, limiting its reproducibility and pharmaceutical development.

To overcome these challenges, a biotechnological approach was employed based on the co-cultivation of *I obliquus* with bacterial cellulose. This method aims to stabilise metabolite synthesis, enhance bioactive compound yield and improve standardisation for pharmaceutical applications. By addressing key limitations in *I obliquus* utilisation, this strategy is proposed for its integration into clinical practice, potentially leading to safer and more effective therapeutic solutions for immune-related and chronic diseases.

Aim of this study was to develop a biotechnological co-cultivation method for *Inonotus obliquus* that enhances metabolite stability and yield, facilitating the production of standardised pharmaceutical formulations.

Methods

Experimental design

A schematic representation of the experimental design is shown in Figure 1.

Research object

The *Inonotus obliquus* strain was sourced from the culture collection of Sechenov First Moscow

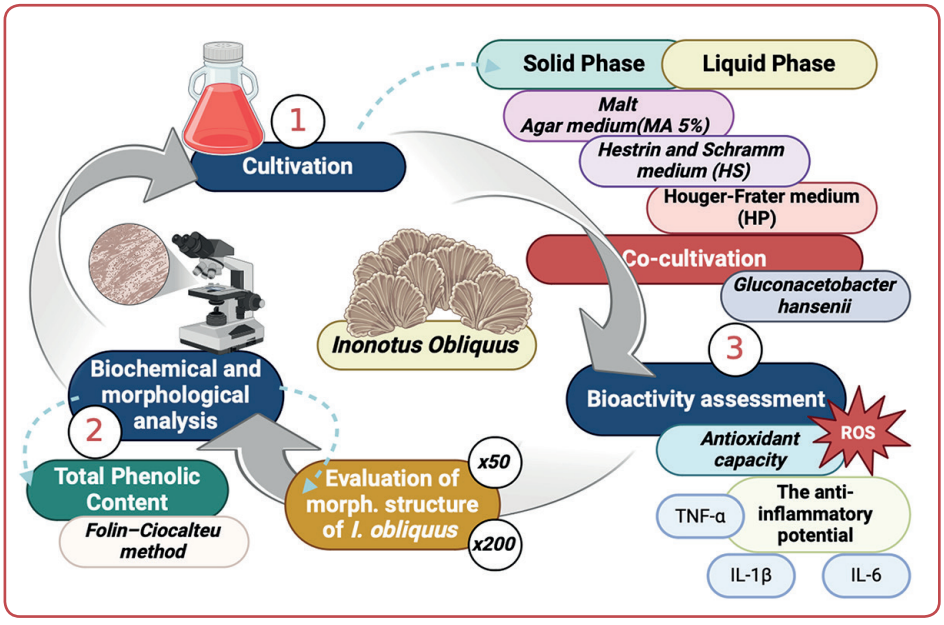


Figure 1: Schematic representation of the study design

State Medical University, Moscow and originally isolated from fruiting bodies on *Betula platyphylla subsp. mandshurica* in the European region, ensuring its natural origin.

Culture media

For the cultivation of *Inonotus obliquus*, several nutrient media were prepared to provide optimal conditions for fungal growth:

- Malt agar (MA 5 %): Non-hopped barley malt extract (5 %) and agar-agar (2.5 %).
- Hestrin and Schramm (HS) medium (g/L): Sucrose (70), yeast extract (5), Na_2HPO_4 (2.7), K_2HPO_4 (2), $(NH_4)_2SO_4$ (3), citric acid monohydrate (1.15).
- Houger-Frater (HP) medium (%): Glucose (2), $(NH_4)_2SO_4$ (0.3), KH_2PO_4 (0.2), $MgSO_4{\cdot}H_2O$ (0.2), ethanol (96 %/2 mL), brewing wort (0.5).

All media were sterilised at 121 °C and 1 atm for 30 minutes using an autoclave.

Cultivation of *Inonotus obliquus*

Inonotus obliquus was maintained on malt agar (5 %) at 4 °C after a 14-day incubation at 27.5 °C. For solid-phase cultivation, it was grown on MA, HS and HP media, with growth assessed on days 3, 5, 7 and 10. Liquid-phase cultivation was conducted in Erlenmeyer flasks containing 50 mL of sterilised HS or HP media, with fungal blocks (1 cm^2) inoculated and incubated at 27.5 °C for up to 21 days. Co-cultivation with *Gluconacetobacter hansenii* involved adding a 3 mL bacterial inoculum to HS and HP media before fungal inoculation, with identical incubation conditions.

Biomass separation and extraction

Fungal biomass was separated from the culture liquid by filtration using a Büchner funnel and ash-free filter paper on 7, 14 and 21 days. The collected biomass was dried at 60 °C to a constant mass and biomass yield (M) was calculated as:

$$M = M_{f+BM} - M_f$$

where $\boldsymbol{M_{f+BM}}$ – the mass of the filter with dried biomass (g) and $\boldsymbol{M_f}$ – the mass of the empty filter (g).

Growth dynamics were assessed by measuring biomass yield, mycelial density and growth rates on 3, 5, 7, 10, 14 and 21 days. Colony expansion was quantified using the linear growth rate (LGR) and growth coefficient (GC), calculated as:

$$LGR = \frac{D-d}{t}; \quad GC = \frac{(D-d) \times H \times g}{t}$$

where $\boldsymbol{D}$ – colony diameter (mm); $\boldsymbol{d}$ – the inoculation block diameter (mm); $\boldsymbol{t}$ –cultivation time (days); $\boldsymbol{h}$ – colony height (mm) and $\boldsymbol{g}$ – colony density (1 = sparse, 2 = moderate, 3 = dense).

Biochemical analysis

The total phenolic content was determined using the Folin–Ciocalteu method, with absorbance measured at 735 nm.[15] A calibration curve was constructed using gallic acid within the concentration range of 25–200 µg/mL.

Morphological evaluation

The morphological structure of *I obliquus* and its biomass were examined using light microscopy at 50× and 200× magnification. Sample slides were prepared by placing fungal fragments in sterile water and covering them with a glass coverslip for direct observation.

Bioactivity tests

The antioxidant capacity of the extracts was assessed using DPPH (2,2-diphenyl-1-picrylhydrazyl) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) assays, with results expressed as IC_{50} values, indicating the concentration required to reduce radical activity by 50 %. Anti-inflammatory potential was evaluated by measuring IL-6, TNF-α and IL-1β levels in LPS-stimulated THP-1 macrophages treated with extracts, using commercial ELISA kits *(BioLegend*, San Diego, CA, USA).

Statistical analysis

Statistical analysis was conducted using SPSS v.12 (*IBM Analytics*, Armonk, NY, USA). Data were presented as mean ± SD. Normality was tested with the Shapiro–Wilk test. Parametric data were analysed using one-way ANOVA with Tukey's post-hoc test, while non-parametric data were assessed *via* the Kruskal–Wallis test with Dunn's adjustment. Cytokine levels were evaluated using two-way ANOVA with Bonferroni correction and non-linear regression was applied to bioactivity assays. Statistical significance was set at $p \leq 0.05$.

Results

Growth parameters of *I obliquus* under solid-phase cultivation

Solid-phase cultivation of *Inonotus obliquus* on MA (5 %), HS and HP media resulted in distinct colony morphologies after 10 days. Fluffy, greyish-beige

mycelium formed on MA, while HS produced a denser, uniform layer. The most compact mycelial structure was observed on HP, where the substrate was completely covered (Figure 2.1).

Growth dynamics were assessed on days 3, 5, 7 and 10 (Figure 2.2). By day 10, colonies reached 1 mm on MA and HS and 2 mm on HP. Linear growth rates ranged from 7.7–10.7 mm/day (MA), 7.4–9.0 mm/day (HS) and 3.1–6.7 mm/day (HP), with peak growth coefficients on day 7 (18.1 for MA, 17.9 for HS, 4.6 for HP). Complete substrate coverage on MA and HS confirmed their suitability for solid-phase cultivation (Figure 2.3).

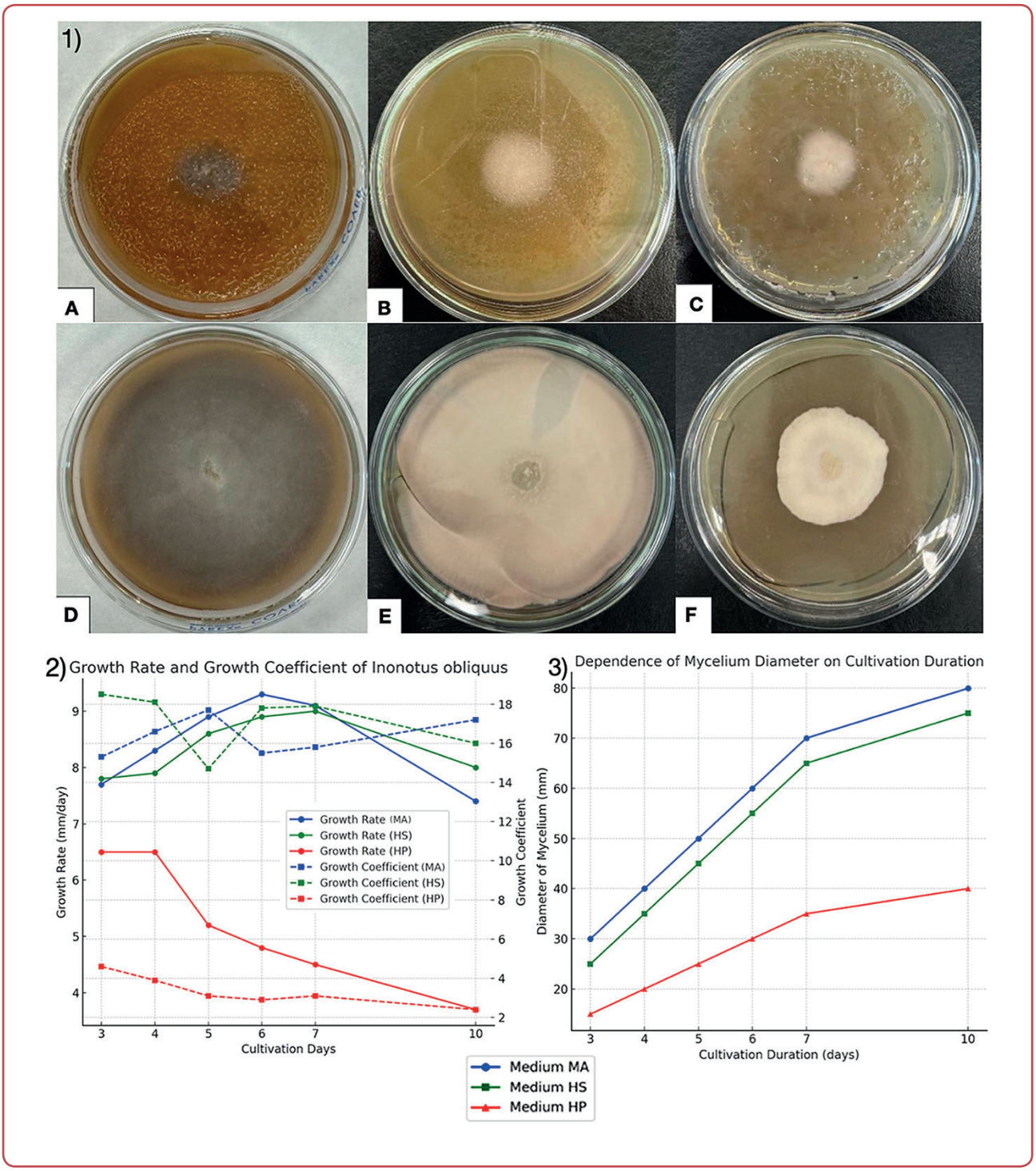


Figure 2: Morphology, growth dynamics and mycelial diameter of Inonotus obliquus during solid-phase cultivation: (1) Mycelial appearance at different time points—day 3 on Malt agar - MA (A), Hestrin and Schramm medium - HS (B), Houger-Frater medium - HP (C); day 10 on MA (D), HS (E), HP (F); (2) Linear growth rate (LGR, mm/day) and growth coefficient (GC) measured at 3, 5, 7 and 10 days on MA, HS and HP; (3) Mycelial diameter (mm) over the same period. Data are presented as mean;

Growth parameters of *I obliquus* under liquid-phase cultivation

Liquid-phase cultivation of *Inonotus obliquus* on HS and HP media at 27.5 °C for up to 21 days revealed distinct mycelial morphologies: HS promoted a dense, cream-colored filamentous network, while HP produced a looser, wool-like structure (Figure 3.1). Biomass yield was highest in HS medium, reaching 17.2 g/L at 21 days, whereas HP yielded significantly less, with 4.6 g/L at 14 days and 4.8 g/L at 21 days. These results indicate that HS is more suitable for liquid-phase cultivation, supporting higher biomass production (Figure 3.2; 3.3).

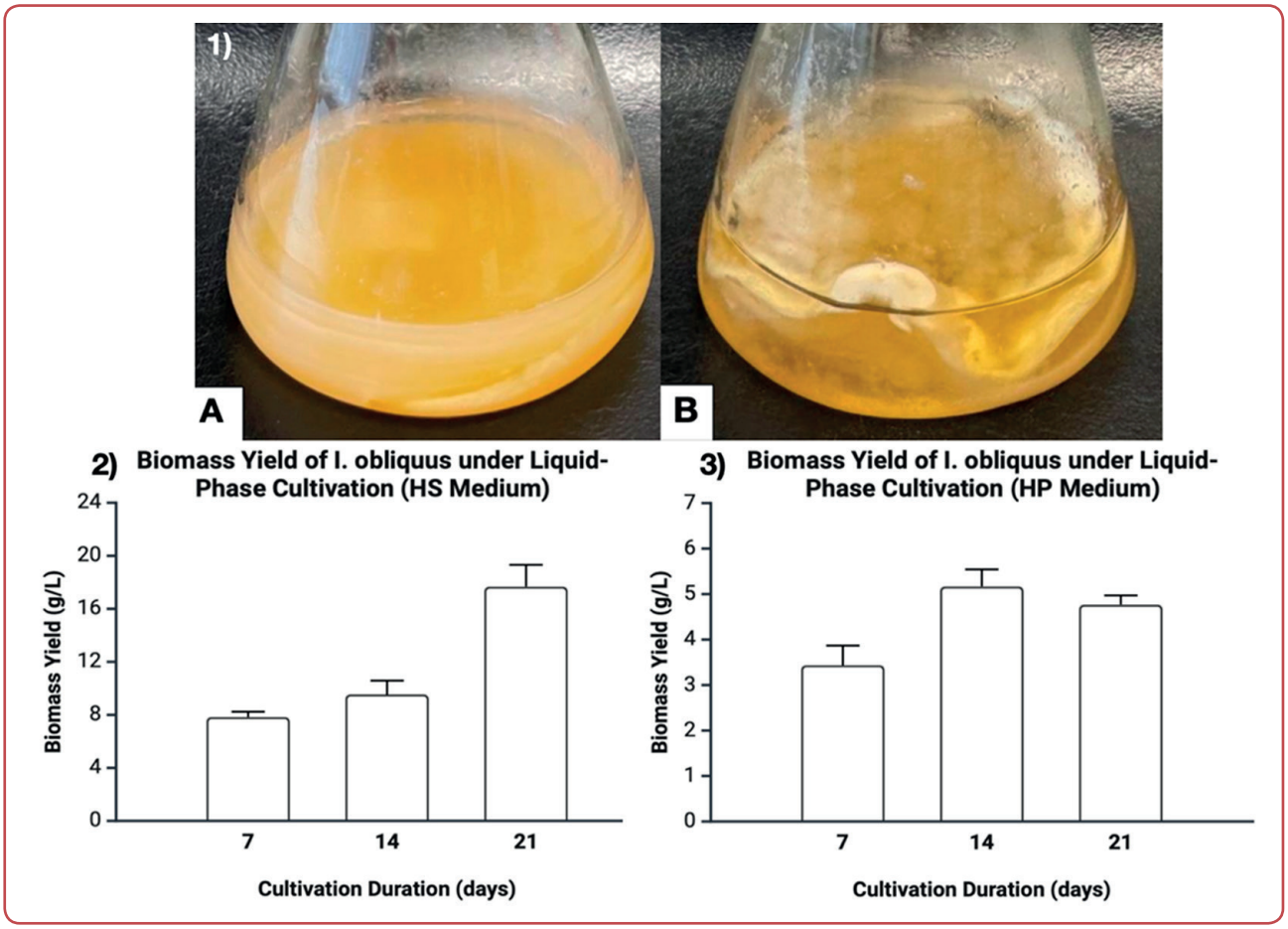


Figure 3: Morphology and biomass yield of Inonotus obliquus in liquid-phase cultivation: (1) Mycelial morphology on Hestrin and Schramm medium - HS (A) and Houger-Frater medium - HP (B); (2) Biomass yield on HS at 7, 14 and 21 days; (3) Biomass yield on HP at the same time points. Data are presented as mean ± SD;

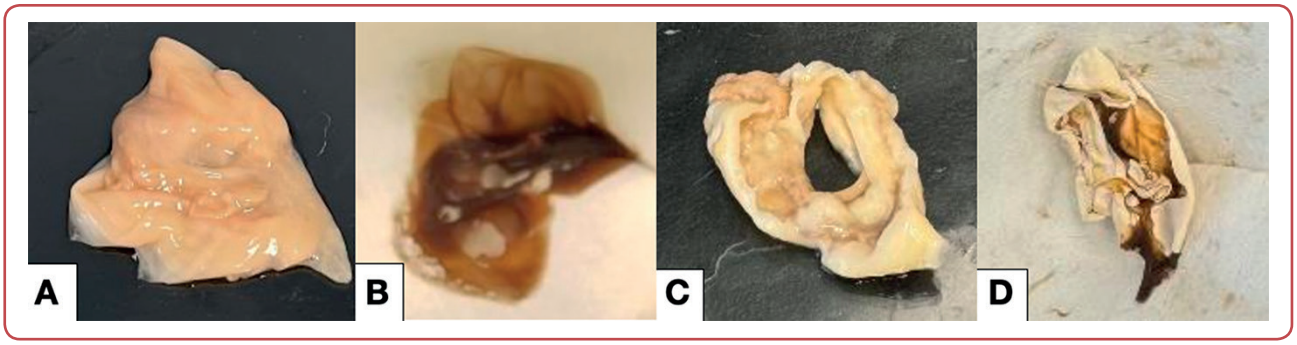


Figure 4: Biofilm formation of Inonotus obliquus mycelium on Hestrin and Schramm medium - HS (A – before drying, B – after drying) and Houger-Frater medium - HP (C – before drying, D – after drying) after 21 days;

During cultivation on HS, the fungal biofilm was thin, smooth and compact. In contrast, HP promoted a rough, uneven biofilm with a central fissure and ring-like formations, suggesting potential fruiting body development (Figure 4).

Growth parameters of *I obliquus* during co-cultivation with *Gluconacetobacter hansenii*

The interaction dynamics and biomass yield of *Inonotus obliquus* and *Gluconacetobacter hansenii* were assessed under co-cultivation in HS and HP media at 27.5 °C. In HS, early formation of bacterial cellulose films facilitated fungal immobilisation, whereas no such structured development occurred in HP – indicating weaker fungal-bacterial interaction (Figure 5).

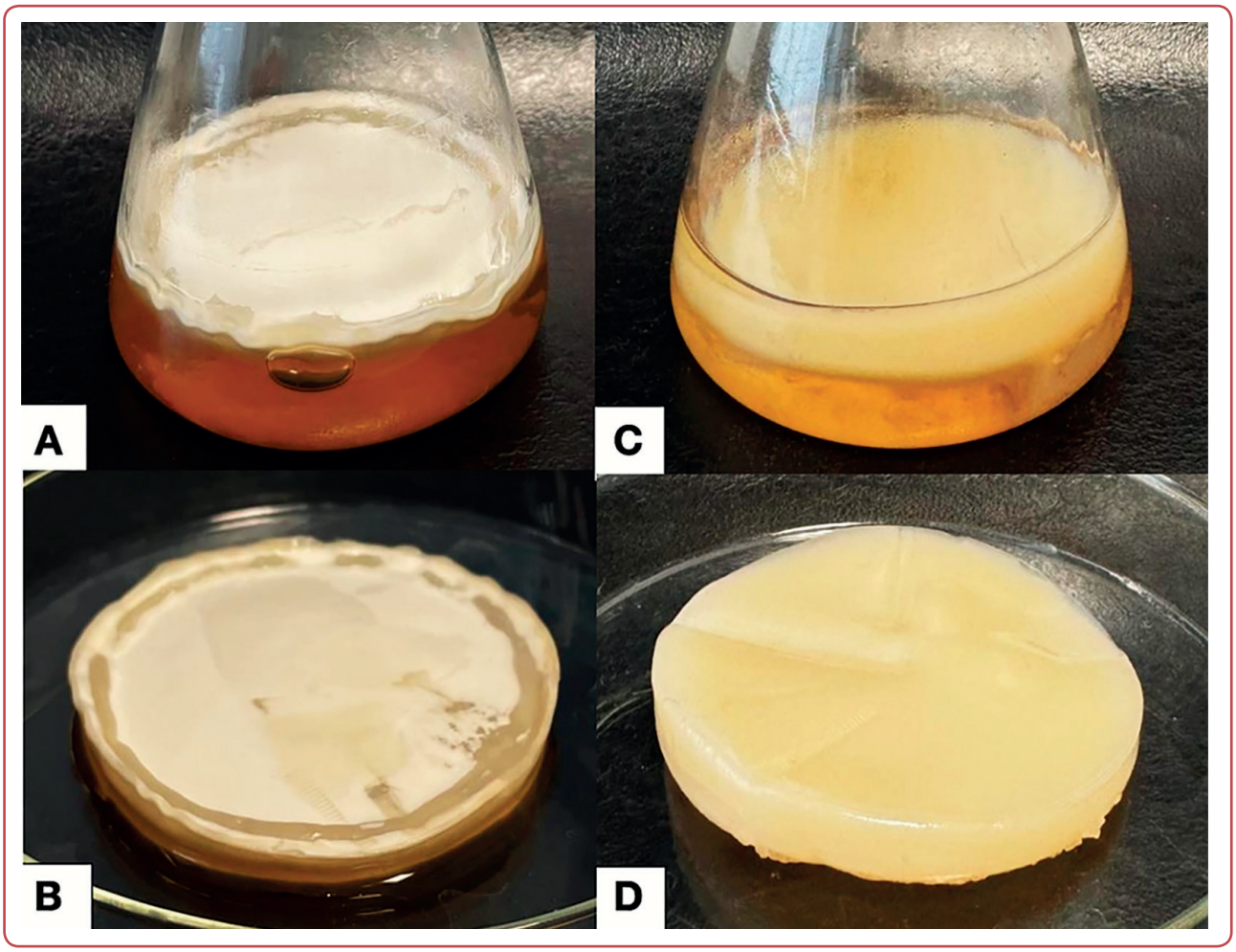
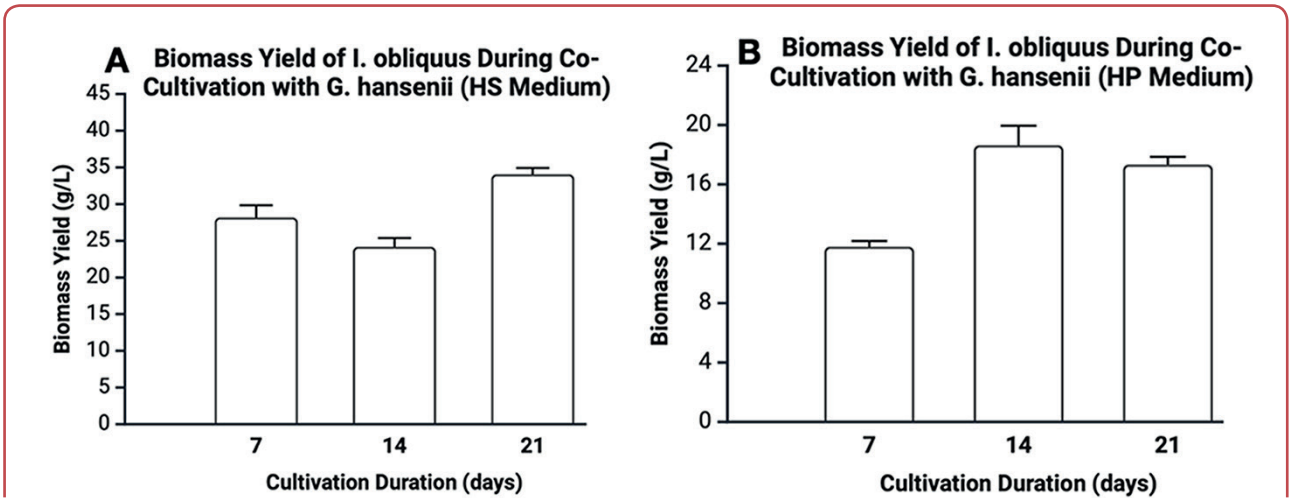


*Figure 5: Immobilised mycelium formation of Inonotus obliquus on bacterial cellulose matrices during liquid-phase co-cultivation with Gluconacetobacter hansenii; (**A, B**) on Hestrin and Schramm medium - HS; (**C, D**) on Houger-Frater medium - HP;*



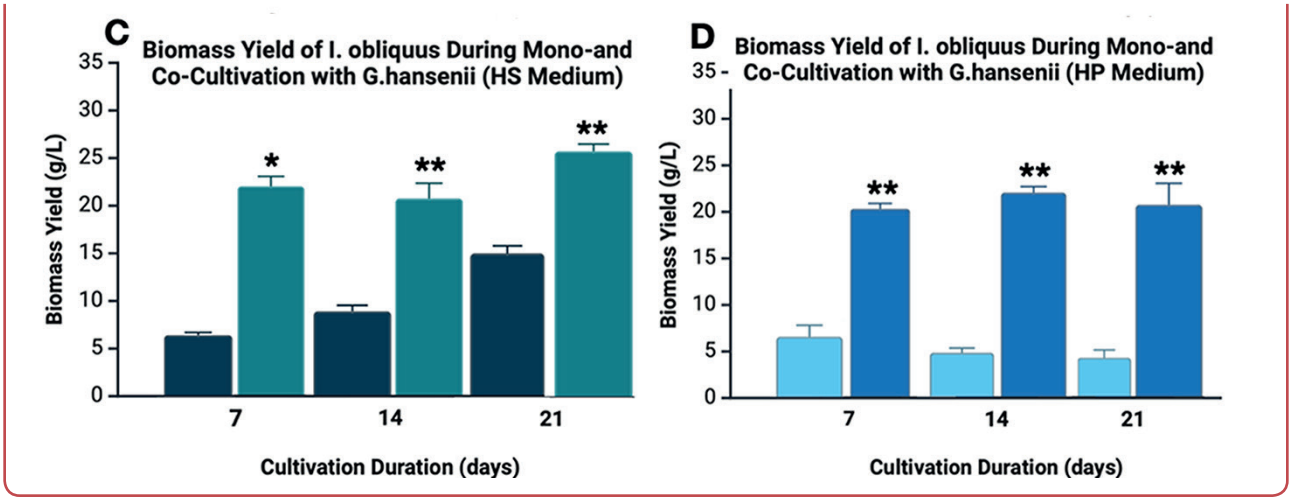


Figure 6: Biomass yield of Inonotus obliquus in mono- and co-cultivation with G hansenii on Hestrin and Schramm medium - HS and Houger-Frater medium - HP at 7, 14 and 21 days: (A) Co-cultivation on HS; (B) Co-cultivation on HP; (C) Biomass comparison in mono- and co-cultivation on HS (monoculture – dark green, co-cultivation – light green); (D) Biomass comparison on HP (monoculture – light blue, co-cultivation – dark blue). Data are presented as mean ± SD. Statistically significant differences from monoculture: $p < 0.05$ (*), $p < 0.01$ (**);

After 21 days, biomass yield peaked at 30.6 g/L in HS, while HP reached 18.2 g/L on day 14, suggesting HS as the more favourable medium for co-cultivation (Figure 6A, B). Compared to monoculture, co-cultivation increased biomass production by 1.8-fold in HS and 3.7-fold in HP (Figure 6C, D). These findings confirm that co-cultivation enhances fungal biomass accumulation, with HS providing optimal conditions for *I obliquus* growth, reinforcing its potential for biotechnological applications.

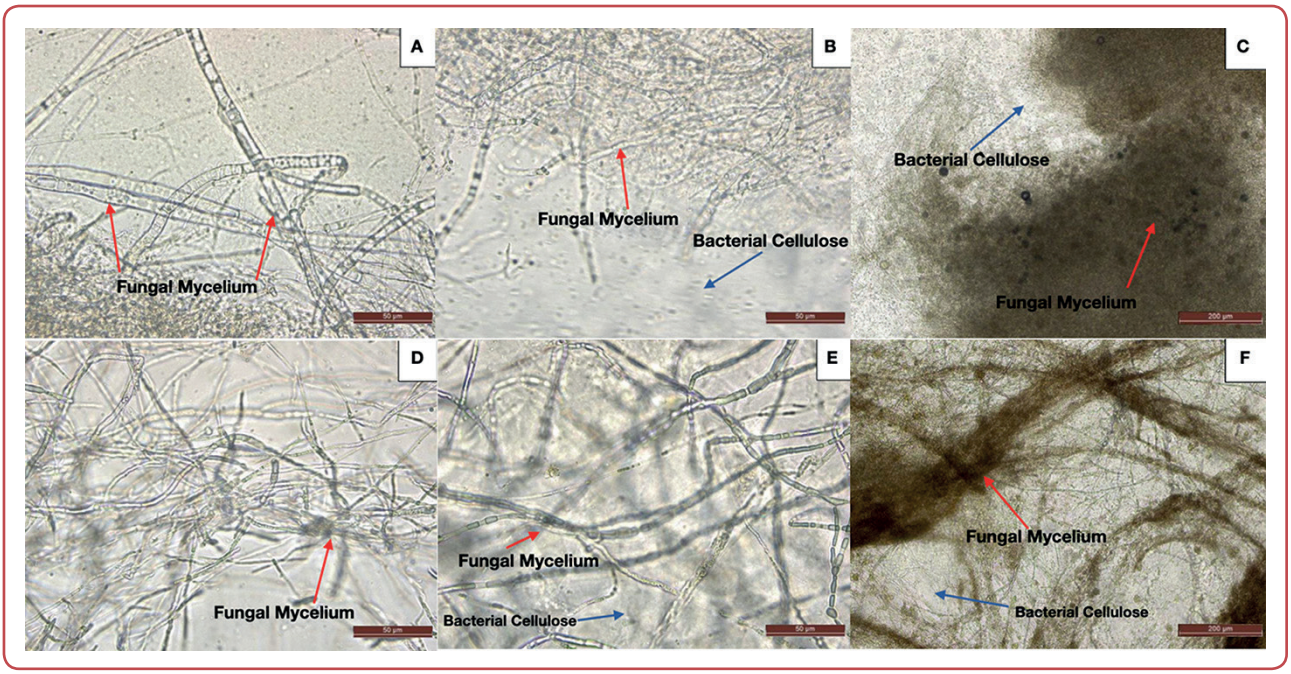


Figure 7: Morphology of Inonotus obliquus mycelium under light microscopy at different magnifications: (A) Monoculture on Hestrin and Schramm medium - HS, ×50; (B) Co-cultivation with Gluconacetobacter hansenii on bacterial cellulose in HS, ×50; (C) Co-cultivation in HS, ×200; (D) Monoculture on Houger-Frater medium - HP, ×50; (E) Co-cultivation with G hansenii on bacterial cellulose in HP, ×50; (F) Co-cultivation in HP; ×200;

Morphological assay

Microscopic analysis revealed distinct morphological differences in *Inonotus obliquus* mycelium between monoculture and co-cultivation. On HS, monoculture produced a dense, uniform hyphal network without voids (Figure 7A), whereas co-cultivation with *Gluconacetobacter hansenii* resulted in structural heterogeneity, with dense hyphal clusters contrasting with lighter cellulose regions (Figure 7B, C).

On HP, monoculture formed a uniform network of elongated, highly branched hyphae (Figure 7D). In co-cultivation, hyphae developed tightly interconnected networks with larger gaps, suggesting bacterial cellulose's role in structural organisation (Figure 7E). At 200× magnification, the cellulose matrix facilitated compact mycelial assemblies with minimal voids (Figure 7F).

Biochemical analysis

Total phenolic content (TPC) of *Inonotus obliquus* extracts from MA, HS and HP media was quantified using the Folin-Ciocalteu method at 735 nm, expressed as mg GAE/g. HS showed the highest TPC (85.4 ± 4.2 mg GAE/g), exceeding MA by 1.3-fold (72.8 ± 3.6 mg GAE/g) and HP by 1.6-fold (64.3 ± 3.1 mg GAE/g) (Figure 8A).

The antioxidant potential of *I obliquus* extracts from MA, HS and HP media was evaluated using DPPH and ABTS assays (Figure 8B, C). HS showed the highest activity (IC_{50}: 43.2 ± 2.5 µg/mL DPPH; 29.8 ± 1.9 µg/mL ABTS), followed by MA (56.4 ± 3.1 µg/mL DPPH; 40.7 ± 2.4 µg/mL ABTS), while HP had the weakest activity (72.5 ± 3.8 µg/mL DPPH; 53.9 ± 2.7 µg/mL ABTS).

Finally, the anti-inflammatory effects of *I obliquus* extracts in LPS-stimulated THP-1 macrophages was assessed by measuring IL-6, TNF-α and IL-1β levels (Figure 8D–F). And HS extracts exhibited the strongest cytokine reduction, followed by MA, while HP showed the weakest effect. These results suggest a correlation between phenolic content and immunomodulatory activity, reinforcing the potential of optimised cultivation for enhancing *I obliquus* bioactivity.

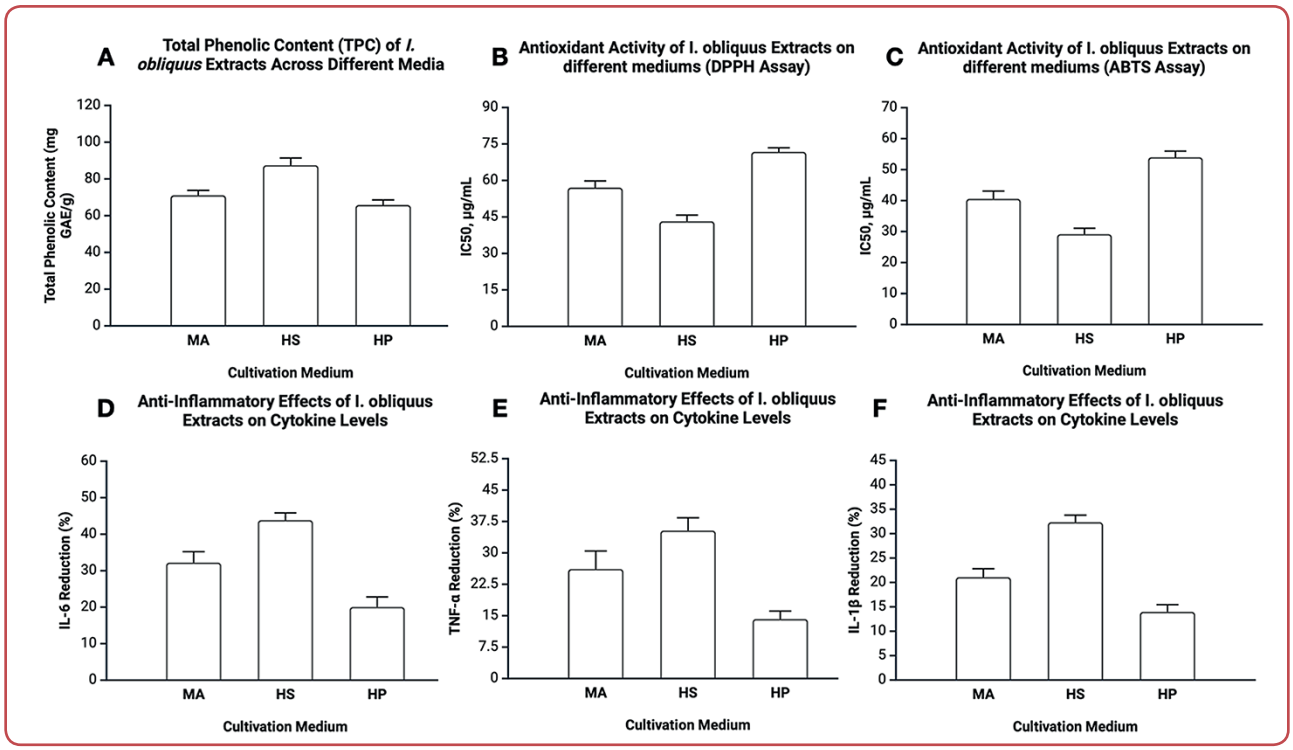


Figure 8: Total phenolic content (TPC) and bioactivity of Inonotus obliquus extracts from different media: (A) TPC measured by the Folin-Ciocalteu method (mg GAE/g); (B) Antioxidant activity (DPPH assay, IC_{50} µg/mL); (C) Antioxidant activity (ABTS assay, IC_{50} µg/mL); (D–F) Anti-inflammatory effects on IL-6, TNF-α and IL-1β in LPS-stimulated THP-1 macrophages, expressed as percentage reduction relative to the LPS-only control. Data are presented as mean ± SD;

Discussion

This study examined how co-cultivation with *Gluconacetobacter hansenii* influences *Inonotus obliquus* growth and bioactivity. Compelling evidence was found that bacterial cellulose enhances mycelial organisation and improves *I obliquus* bioactive potential.

In presented study, co-cultivation with bacterial cellulose enhanced phenolic compound production and stabilised metabolite synthesis in *I obliquus* (an effect likely mediated by its structural role in mycelial organisation and metabolic activity), consistent with prior studies on optimised fungal cultivation.[16] Beyond the enhanced phenolic synthesis, co-cultivation with *Gluconacetobacter hansenii* improved the overall bioactivity of *Inonotus obliquus*, including its antioxidant, anti-inflammatory and immunomodulatory effects. Bacterial cellulose not only enhanced phenolic output but also contributed to greater consistency in metabolite production, likely through its structural impact on mycelial organisation.

A potential explanation for this enhancement lies in the structural rearrangements induced by bacterial cellulose; morphological analysis revealed denser and spatially organised hyphal networks under co-cultivation. While monoculture resulted in uniform hyphal distribution, co-cultivation induced compact clustering, likely due to cellulose-mediated anchoring, consistent with findings by Hoenerloh et al.[17] The increased network complexity likely enhanced nutrient accessibility and stability, highlighting bacterial cellulose as a key factor in optimising *I obliquus* cultivation for scalable bioactive compound production.

In addition, co-cultivation with bacterial cellulose significantly increased the total phenolic content (TPC) in *I obliquus*, with HS medium yielding 85.4 ± 4.2 mg GAE/g—1.6-fold higher than HP medium. This aligns with previous findings by Wang et al, who reported enhanced phenolic biosynthesis under optimised liquid culture conditions.[18] The elevated phenolic levels correlated with stronger antioxidant activity, as demonstrated by DPPH (IC_{50} = 43.2 ± 2.5 µg/mL) and ABTS (IC_{50} = 29.8 ± 1.9 µg/mL) assays, surpassing values previously reported for *I obliquus* extracts.[19] Enhanced phenolic biosynthesis also translated into greater anti-inflammatory potential, as HS medium extracts induced the most pronounced reduction in IL-6 (47.5 %) and TNF-α (42.8 %) levels in LPS-stimulated macrophages. These results are consistent with the immunomodulatory effects of *I obliquus* polysaccharides observed by Wagle et al.[20] The underlying mechanism may involve metabolite diversity promoted by co-cultivation, though further analysis is required to confirm this interaction.

Bacterial cellulose-based co-cultivation enhances *I obliquus* bioactivity, supporting its scalability for industrial applications. However, ensuring reproducibility across environmental conditions remains a challenge.

Taken together, these findings establish co-cultivation with bacterial cellulose as a scalable strategy to enhance the bioactivity of *I obliquus* for industrial purposes. To advance its translational potential, future research should focus on elucidating the underlying regulatory mechanisms of phenolic biosynthesis and linking structural dynamics to metabolite output through integrated physicochemical and metabolomic analyses.

Conclusion

The present study demonstrated that co-cultivation with bacterial cellulose enhances *Inonotus obliquus* mycelial organisation and bioactive metabolite synthesis, particularly phenolics with antioxidant and anti-inflammatory properties. This approach not only offers a scalable and sustainable approach for industrial applications and lays the foundation for future biopharmaceutical development. While these findings highlight its promise for bioactive compound production, further research is needed to optimise cultivation parameters, ensure reproducibility and validate therapeutic efficacy *in vivo*.

Ethics

This study did not directly involve with human participants or experimental animals. Therefore, the ethics approval was not required in this paper.

Acknowledgement

None.

Conflicts of interest

The authors declare that there is no conflict of interest.

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Data access

The data that support the findings of this study are available from the corresponding author upon reasonable individual request.

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Project administration: MAP

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