BioEconomy
Research & Advisory

Unlocking full potential of the biomass:
Producing higher value products
by use of new enzymes & smart technologies

Lene Lange

"LL-BioEconomy, *Research & Advisory*, Denmark"

Research Partner in Danish, Nordic, EU & Internationally-funded projects

Advisory to the Euopean Commission (CBE, Scientific Committee),

to the Danish Government (Naional Bioeonomy Panel)

and to Nordic Council of Ministries, Icelandic Chairmanship (etc etc)

Global Focus:

Improved use of biological resources is a necessity

=> Many new types of improved enzymes are needed

Huge opportunities in enzyme-processing, making Value from ”Waste”:

- **Upgraded** use of *food waste:* Globally, 34% of all food is wasted!
- **Valorizing *food-processing side-streams;*** *currrently >6% extra is wasted; => >40%*
- **Improved use of *crop residues*** *–unlocking full potential of entire crop plant*
- **Sustainable higher value use of *blue biomass*,** *seaweeds & fish cut-offs*
- **Upcycling of *residual microbial biomass*** from biological production /bacteria & fungi
- **Making value from organic content** of *sludge and wastes*
- **Upgraded use of fibers** from *outsorted textiles, for production of new textiles?*

Lange, O´Connor et al., 2021: *Developing Sustainable & Circular biobased Economy in EU. Front.Bioeng.Biotechnol. 2021*

By Cascading use:

Unlocking the full potential of the many types of biomass

- The **Yellow** Biomass: Straw, corn-stover & wood chips
- The **Green** Biomass: Green grass & foliage and stems
- The **Blue** Biomass: Fish processing cut-offs & Seaweeds
- The **Red** Biomass: Slaughterhouse waste, blood & cut-offs
- The **Grey** Biomass: Agro-industrial side streams
- The **Brown** Biomass: Sludge & manure; household waste
- The **Purple** approach: Making feed from methane (Negative Emission Tech)

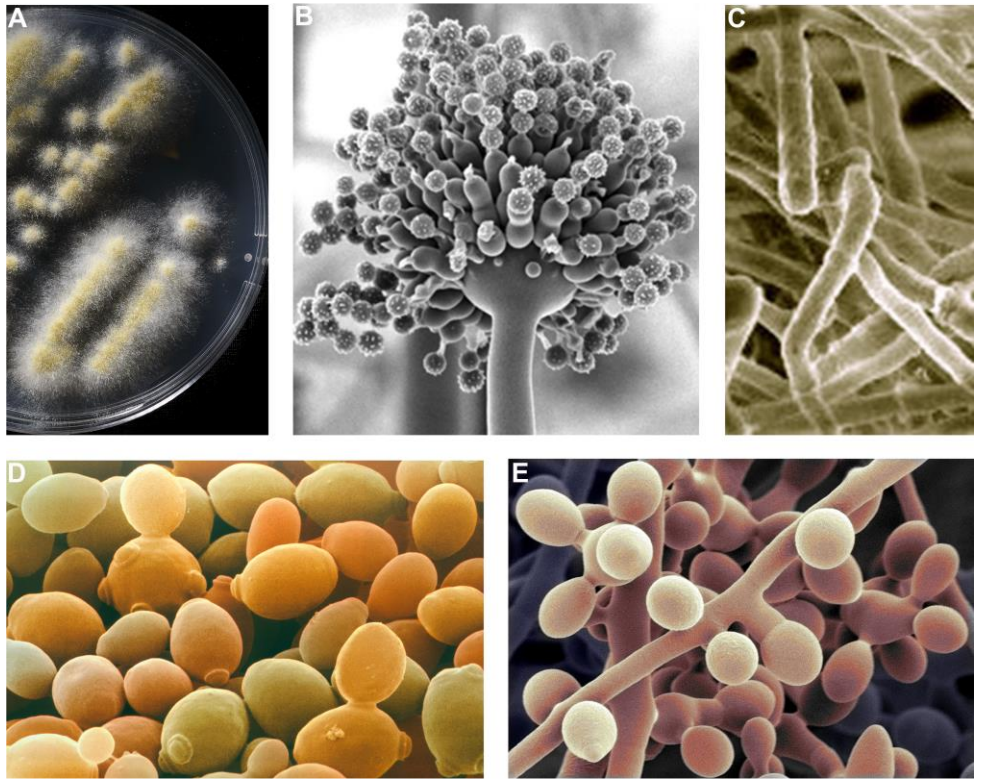
Lene Lange, Infographics, Creative Commons (The Colors of Biomass & Biorefinery)

Learning from Nature

Fungi can play a prominent role for improved use of global biological resources

- Fungal invasive power enables upgrading of the bioresources, now wasted
- Enzymes, Production host, Single cell proteins
- & Higher value products!

Lene Lange, Infographics, Creative Commons
(Fungal Hall of Fame)



Reference:

Lange L, Agger JW, Meyer AS. Fungal Biotechnology: Unlocking the Full Potential of Fungi for a More Sustainable World.
http://link.springer.com/10.1007/978-3-030-29541-7_1

Higher value products from the pulp-fraction of the Green Biorefinery

- **Gut-health promoting food ingredients with prebiotic effect**
  - By enzyme treatment of the hemicellulose, Xylan-containing polymers, producing Xylo Oligo Saccharides, XOS
    - stimulating the healthy microbiome biodiversity

- **Chloroplast-protein, extracted from the green fibrous pulp**
  - by protease enzyme treatment making e.g. plant-based baby food

Outreach-Reference: Lange, L. et al., 2016. The Fundamentals of Bioeconomy for a Biobased Society. United Federation of Danish Workers 3F (available in 8 European languages). https://backend.orbit.dtu.dk/ws/portalfiles/portal/140638164/

Higher value products from the Blue biorefinery

Pharma and health products from fish skin & from shrimp shell processing

- Wound healing (Penzyme, Enzymatica)
- Common Cold (Coldzyme, Enzymatica)
- Collagen (Codland, Iceland)

- **Pharma/wound healing compounds, pigments & enzymes from seaweeds**
  - Prophylaxis and treatment of Age-inferred eye-disease, MDA
    - A Fucoidan-based product

- Reference: Lange, L. et al., 2021. The Blue Bioeconomy. United Federation of Danish Workers 3F (available in English, Danish and Icelandic; Greek in prep).

Higher value products from the Red Biorefinery

- Pharma product, blood-based
  - for treatment of iron-deficiency in women and children
- New Enzymes, discovery and use
- High quality proteins for food and feed from poultry
  - Circular use of clean slaughterhouse sidestreams
  - -now OK for poultry waste (no BSE!)

References: **Espersen R**, Huang Y, Falco FC, Hägglund P, Gernaey KV, **Lange L**, et al. 2021. Exceptionally rich keratinolytic enzyme profile found in the rare actinomycetes *Amycolatopsis keratiniphila* D2T. Appl Microbiol Biotechnol. 105(21):8129–38. **Lange L, Huang Y, Busk PK**. Microbial decomposition of keratin in nature—a new hypothesis of industrial relevance. Appl Microbiol Biotechnol. 2016, 100(5):2083–96

Higher value products from cereal food-processing side-streams

- **Deliciousness** can be the new driver of climate friendly food:
  - By specific enzyme treatments and gourmet processing*:
  - Upgrading cereal bran to Umami-flavored Sauce or Umami-flavored Bouillon
    - For enrichment of taste of plant-based food

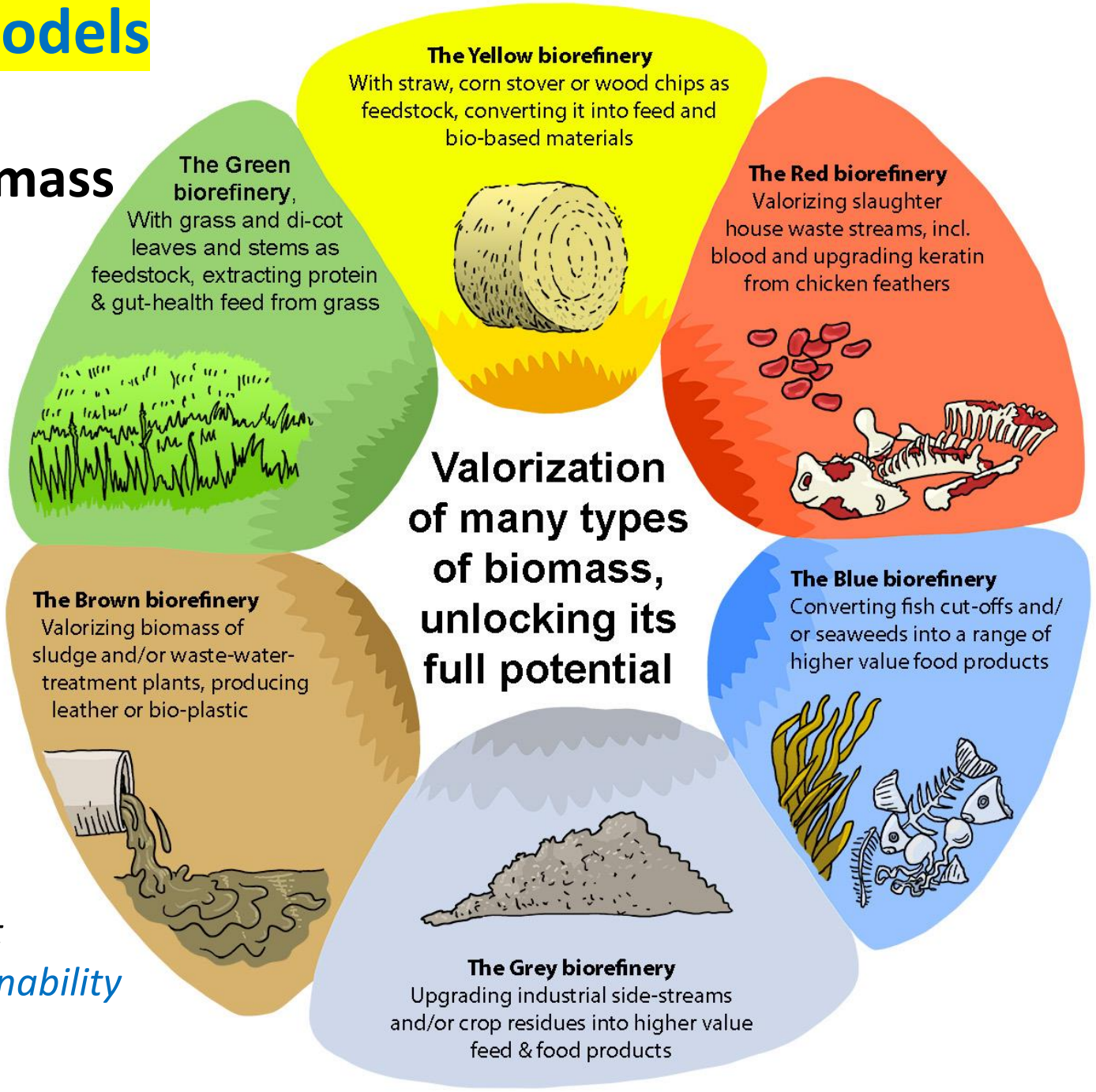
- Speed up, making "**The green choice = The preferred choice**"!

**** Made by "Amass R&D", in collaboration with "LL-BioEconomy"***

Bio-based Business Models

For Upgraded use
of the many types of biomass

To implement producing also high-value products we need many new and improved enzymes!



Lange, L., 2022: *Business Models, including higher value products for the new circular, resource-efficient bio-based industry, Frontiers in Sustainability*

Technologies for improved Enzyme Discovery:
Developing Peptide-based Functional Annotation

- New technologies, described by analysis of genome sequenced fungi

- Notably, all technologies also applicable for other types of organisms (bacteria, plants, animals etc)

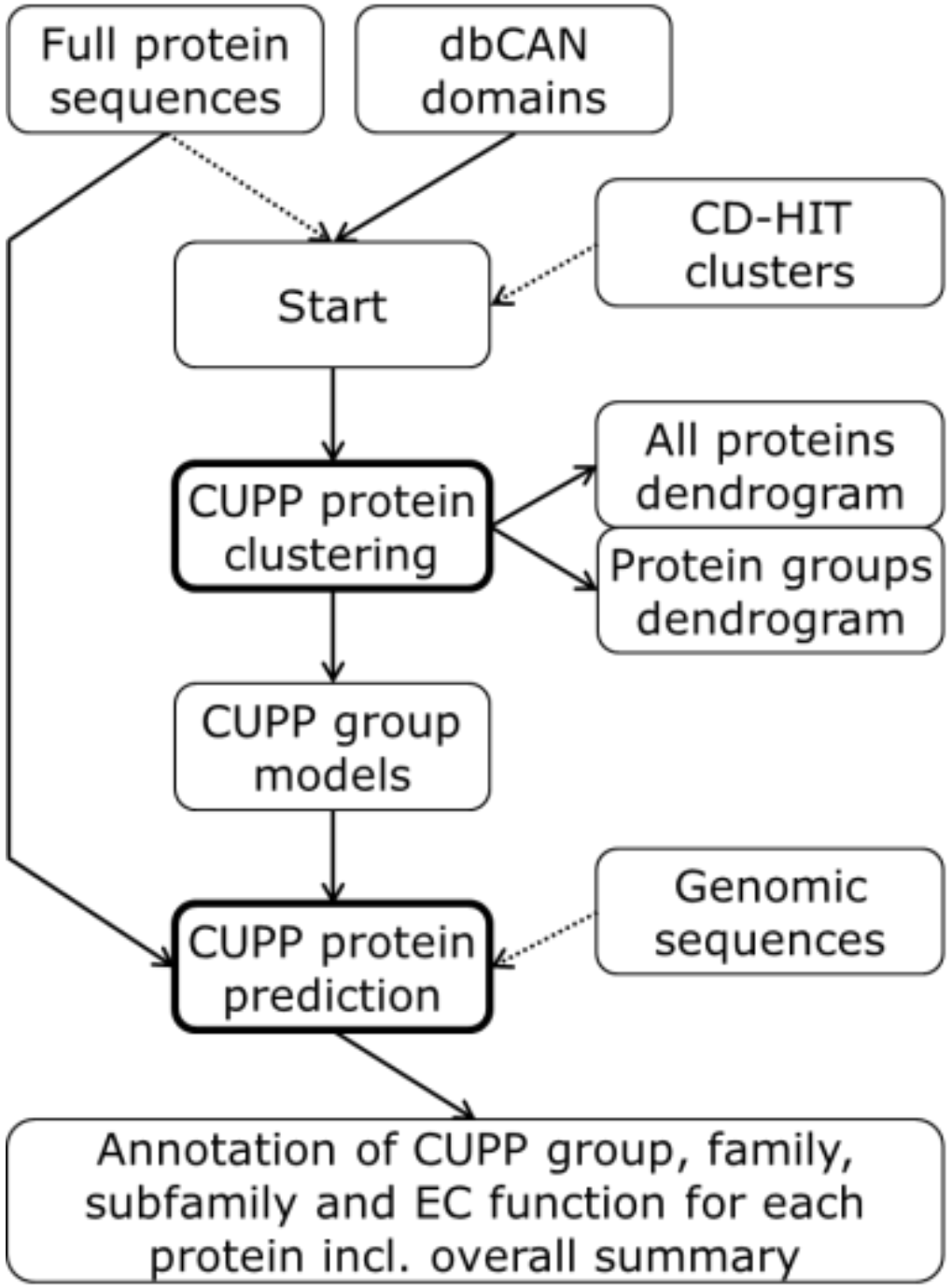
Functional annotation by CUPP, Conserved, Unique Peptide Patterns

CUPP for CAZymes:

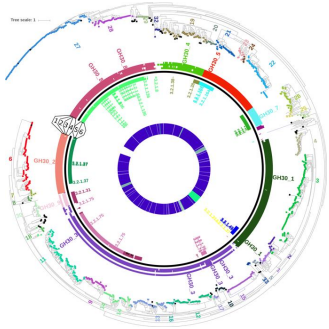
Consists of two stages,
Clustering & ***Function Prediction***

Barrett & Lange*, 2019
in Biotech f Biofuel:
CUPP, Peptide-based Functional annotation

Barrett et al., 2020
CUPP as Online functional annotation platform.
Nucleic Acid Research, 48, p110-115 **TRY IT!**



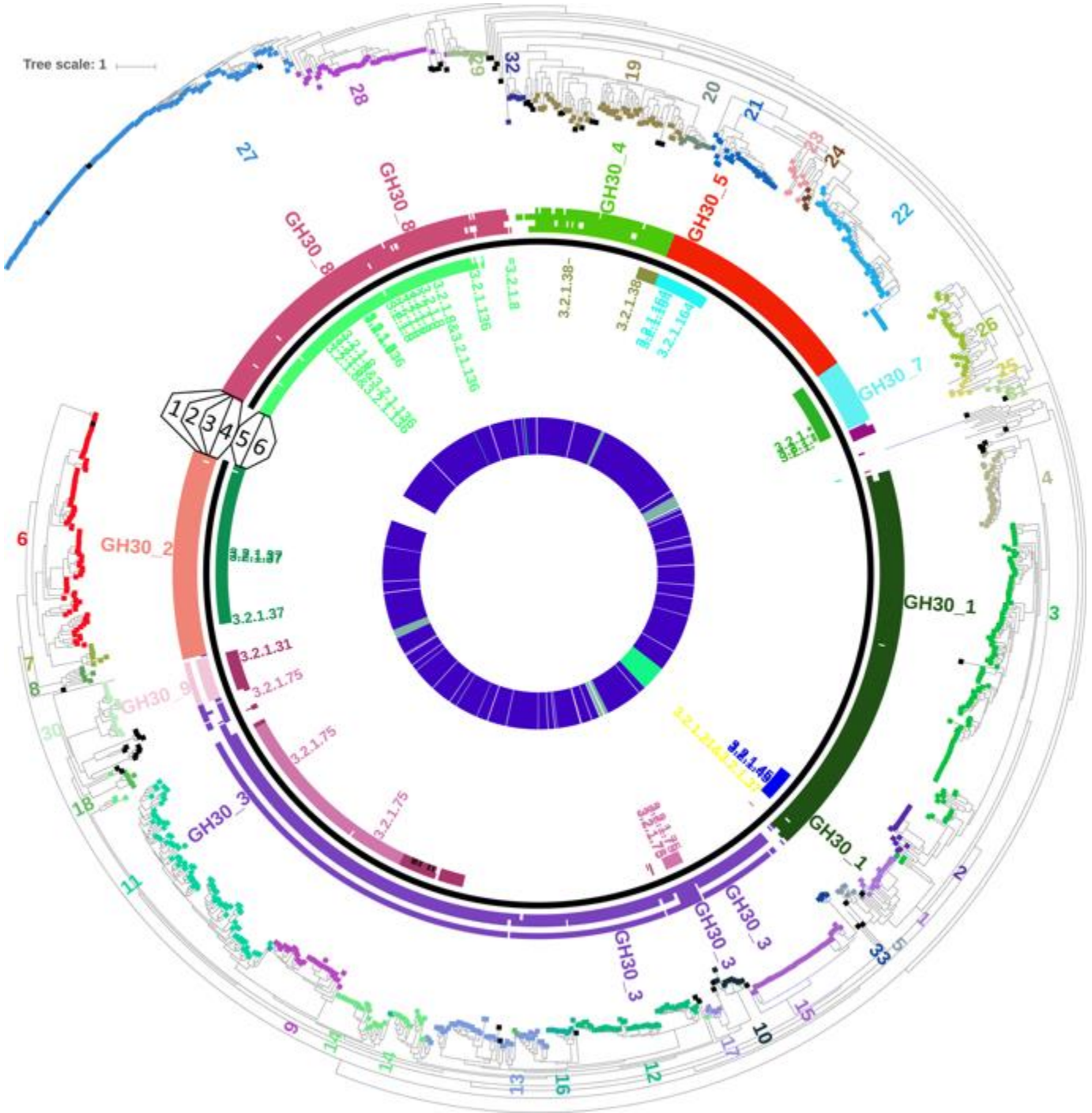
Validation of CUPP functional annotation (ex: CAZyme Family GH30)



- In general, each CAZy Family holds several enzyme functions
- CUPP groups represent a lower level division than CAZyme Families/ Subfamilies
- Members of a CUPP group have same EC Function (or share other function-relevant features)
- CUPP-prediction of function optimizes use of data/information: *Prediction of function possible, if just one member of a CUPP group has been characterized*

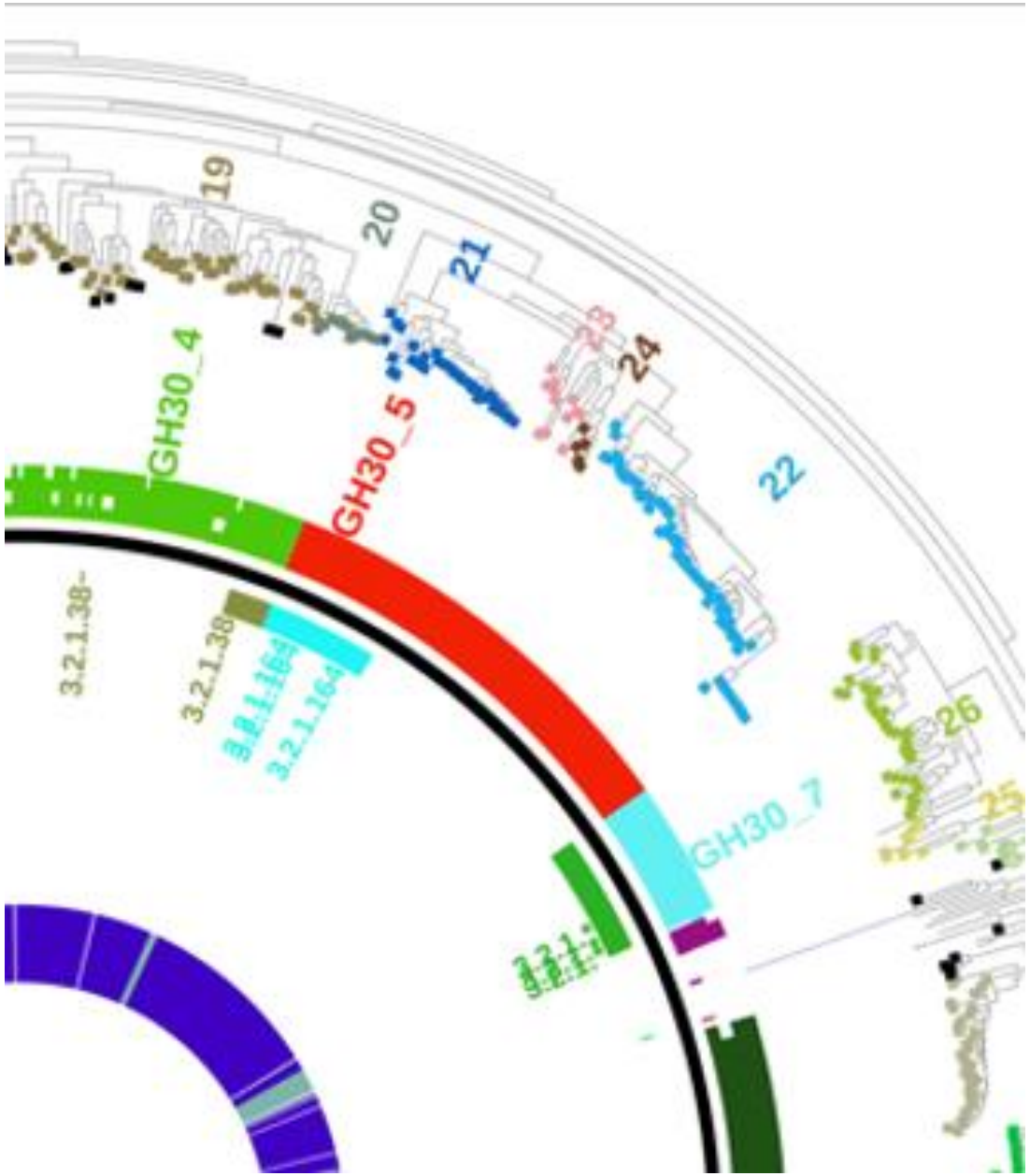
CUPP validation on GH30:

Case, all proteins belonging to glycohydrolase family GH30



Grouping proteins of CAZy Family GH30 into Families & SubFamilies

Surrounding dendrogram indicates division of Subfamilies into CUPP-Groups



GH30_5:
GH30, Sub-family 5 is divided into CUPP-Groups 21-24

Prediction of function possible, if just one member of a CUPP group has been characterized

Improvements of peptide-based functional annotation -from PPR/HotPep to CUPP *(now an online web-based tool)*

CUPP:

- All groups formed simultaneously=> Gr#1 no longer disproportionally large
- CUPP Group numbers conserved; allowing comparing results over time
- Using Unique peptide patterns for each CAZyme CUPP group –no overlap
- Improved Sensitivity and Precision in Prediction of Function
- Basic principle covered by same, open-access patent (Busk & Lange)
- CUPP available as automated online platform https://cupp.info/

- ***Busk, PK & Lange, L, 2013:*** *Function-Based Classification of Carbohydrate-Active Enzymes by Recognition of Short, Conserved Peptide Motifs. Appl Environ Microbiol. 79: 3380–3391*
- ***Barrett, K & Lange, L, 2019****: Peptide-based functional annotation of carbohydrate-active enzymes by conserved unique peptide patterns (CUPP). Biotechnology for biofuels 12 (1), 1-21. CUPP method, description and validation*
- ***Barrett, K et al., 2020****: Conserved unique peptide patterns (CUPP) online platform: peptide-based functional annotation of carbohydrate active enzymes, Nucleic Acids Research, 48, p110-115. Try it! It is online!*

CUPP for enzyme discovery

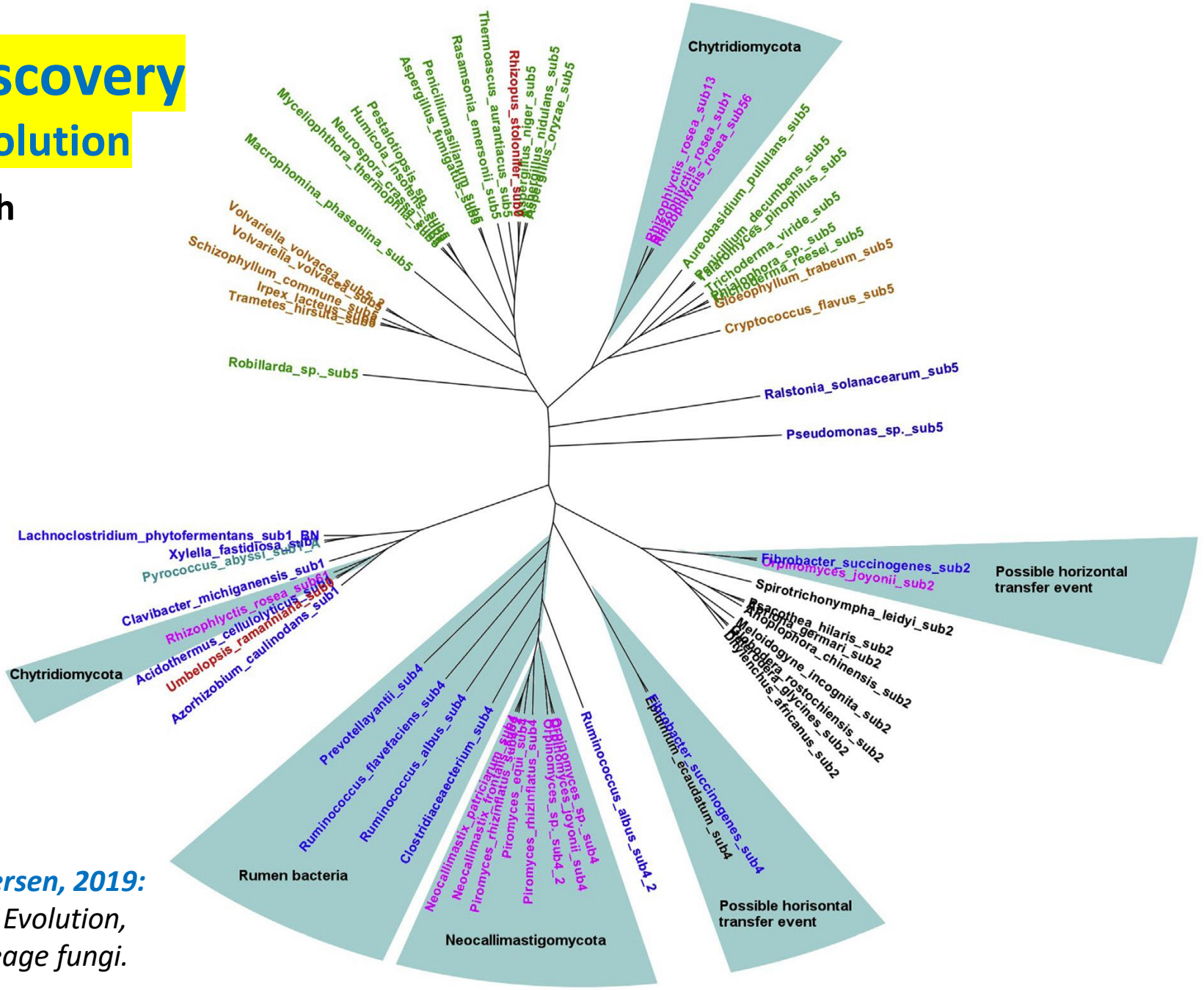
- elucidating secretome evolution

Ancient zoosporic fungi have rich secretome and unique enzymes
Case endo-glucanase GH5,
Phylogenetic tree:

Ascomycota
Basidiomycota
*Chytridiomycota -all over!
Zygomycota

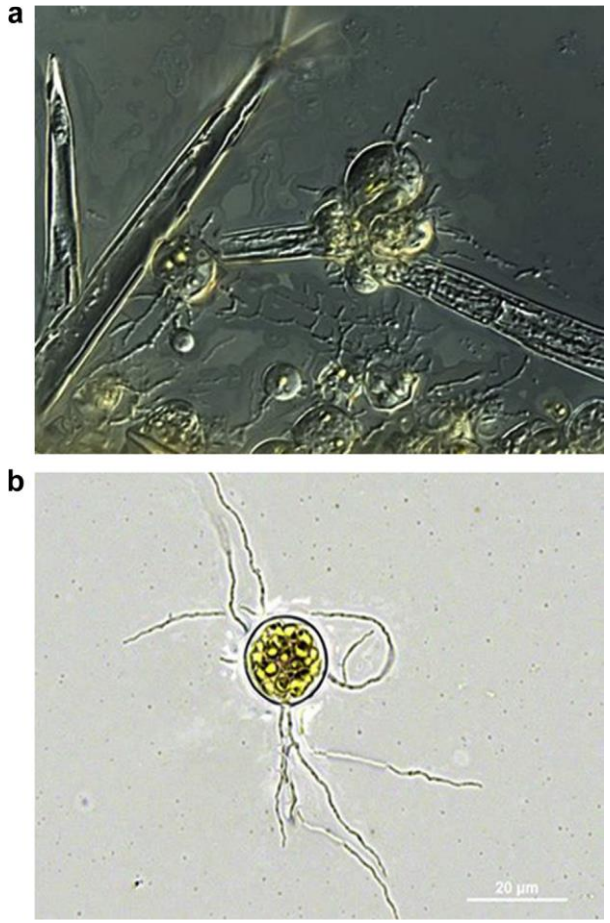
Bacteria
Archaea

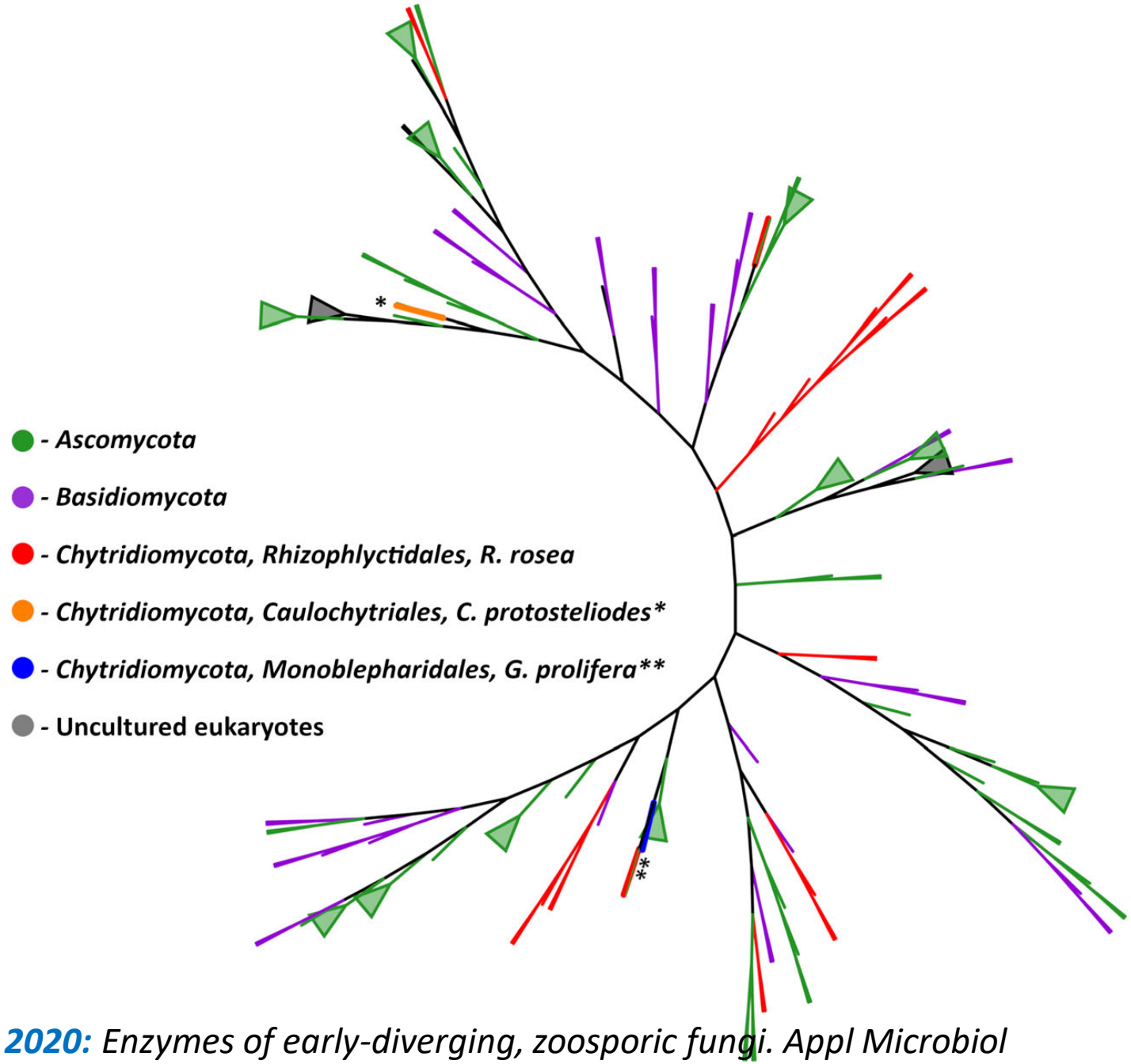
Plants
Animals



Lange, Pilgaard, Herbst, Busk, Gleason, Pedersen, 2019:
Origin of fungal biomass degrading enzymes: Evolution, diversity and function of enzymes of early lineage fungi. Fungal Biology Reviews, 33: 82-97

Phylogenetic tree, fungal AA9:
Zoosporic, *Rhizophlyctis rosea* a soil chytrid, has rich and unique diversity of AA9 LPMOs (cellulose)





Lange, L., Barrett, K., Pilgaard, B., Tsang, A., 2020: *Enzymes of early-diverging, zoosporic fungi. Appl Microbiol Biotechnol* ***103,*** *6885–6902. https://doi.org/10.1007/s00253-019-09983-w*

New: CUPP-based secretome-annotation for enzyme discovery

Basic principle of functional secretome annotation:

Integrate Protein-Family & EC-Function into one ”Function;Family Observation” ”F;F”

Secretome composition of ”F;F” is the target for Evolutionary pressure & selection:

- **Fitness: Having the needed types of digestive functions in right types of proteins**
  - *Protein structure determines substrate accessibility, stability, pH- and temperature- tolerance and optimum (etc)*

Annotated ”Function;Family” profiles mimick evolutionary fitness:

- Optimized secretome mobilizes the substrate efficiently, hereby giving basis for fitness in growth and reproduction

***Barrett, Jensen, Meyer, Frisvad*, Lange*, 2020**: Fungal secretome profile categorization of CAZymes by function and family corresponds to fungal phylogeny and taxonomy: Example Aspergillus and Penicillium. Scientific Reports*

EPR-Hypothesis:

Similarity in ”F;F” annotated secretome profiles matches phylogenetic relatedness of the producing organisms

Enzyme profile relatedness (EPR) can be found by binary comparison of ”F;F”composition

Validation model:

All available genome sequenced *Aspergillus* and *Penicillium*

- Testing by Jacquard calculation: Did not confirm hypothesis!
- **Testing by Yule distance calculation confirmed the hypothesis!**
  - ***Yule*** *gives equal weight to F;F observations* ***shared*** *& F;F observations* ***shared*** *NOT having (presence and absence)*

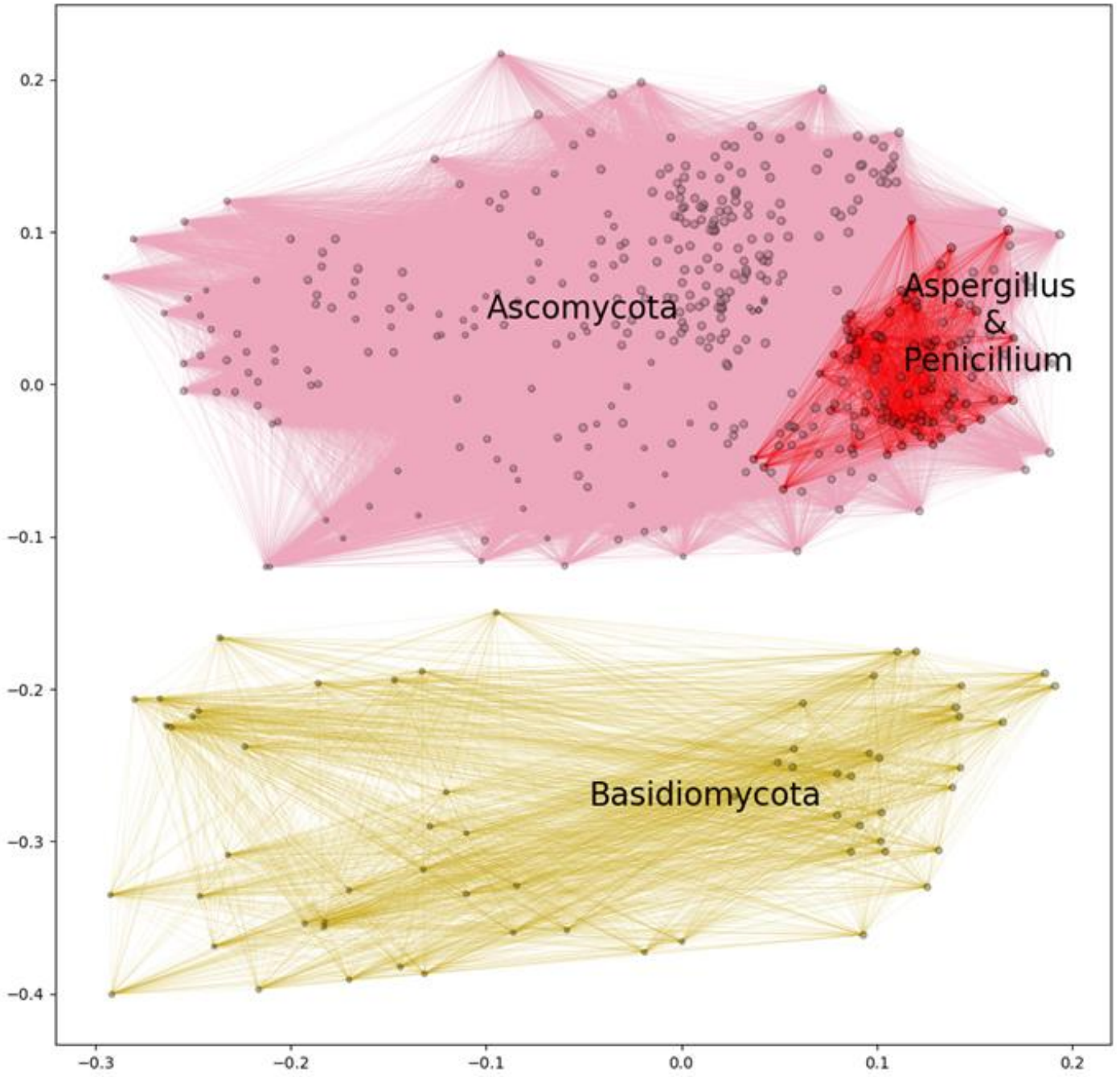
Barrett, Jensen, Meyer, Frisvad*, Lange*, 2020*: Fungal secretome profile categorization of CAZymes by function and family corresponds to fungal phylogeny and taxonomy: Example Aspergillus and Penicillium. Scientific Reports*

Enzyme Profile Relatedness-based clustering of genomes across Fungal Kingdom

By Multi-Dimensional-Scaling:

***Distinct separation of Asco- from Basidiomycota!**

Distinct clustering of *Aspergillus- & Penicillium spp



Barrett, Jensen, Meyer, Frisvad, & Lange, 2020:
Fungal secretome profile categorization of CAZymes by function and family corresponds to fungal phylogeny and taxonomy: Sci Rep 10, 5158

Testing Hypothesis: EPR-clustering of all species of Penicillium and Aspergillus (=molds)

Integrating:

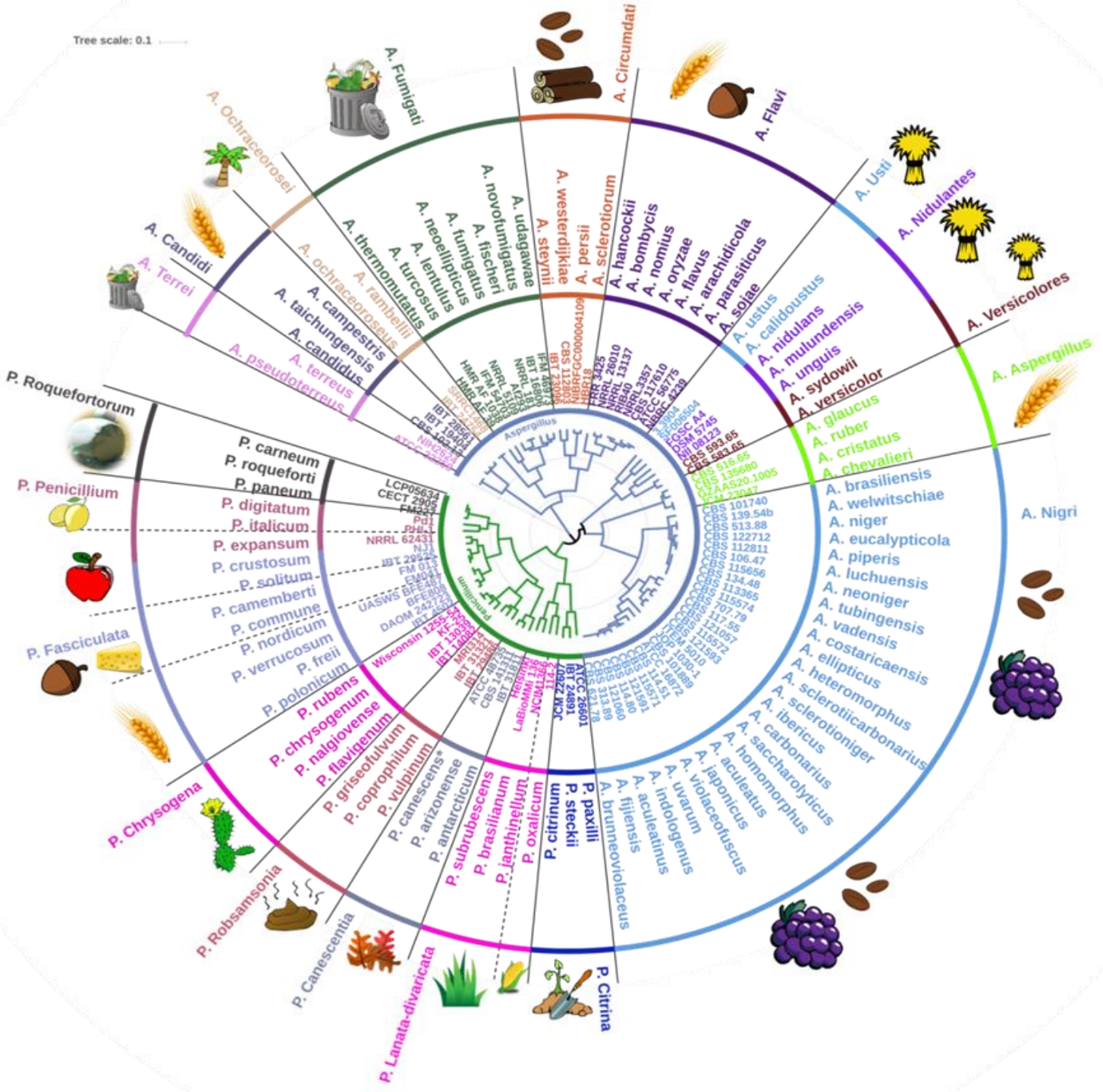
*Functional genomics EPR dendrogram /center

*Organismal phylogeny

*Interaction-phenotype (substrate affinity=icons)

Result:

Complete match of EPR to division of species of the two fungal genera into Sections



NEW: Fungal Kingdom analysis, finding the Enzyme ”Hotspots”
Basic principle: Summing up number of ”F;F” observations!

- **Summing up ”F:F” observations per species, allows for ranking of all Fungal Kingdom genome sequenced species (~2.000), according to:**
  - Richness in CAZyme function-specificity diversity (only unique ”F;F” obs.)
  - Total degrading capacity (incl redundant ”F;F” obs.)

- **Analyzing for fungal CAZyme Hotspots identifies:**
  - Fungal species ”richest & poorest” in cellulolytic, xylanolytic, pectinolytic & ligninolytic enzyme potential

- **The fungal enzyme Hotspots are found in very different types of fungi:**
  - Many surprises: e.g. hot spots found among endophytes and stone-fungi
  - Strongest degrading capacity: in zoosporic rumen fungi

Lange, L.; Barrett, K.; Meyer, A.S., 2021: *New Method for Identifying Fungal Kingdom Enzyme Hotspots from Genome Sequences. J. Fungi 7, 207*

Hot Spot analysis, example

10 top-scoring fungal species, ranked by pectin-degrading capacity

D Ranking: Pectin	Taxonomy		Target Substrate of Encoded CAZymes				
Species	Class	Phylum	Cellulose	Pectin	Xylan	Lignin	Total
Mycena citricolor	Agaricomycetes	Basidiomycota	91	204	50	149	494
Verticillium longisporum	Sordariomycetes	Ascomycota	139	176	74	95	484
Paramyrothecium roridum	Sordariomycetes	Ascomycota	106	163	63	79	411
Colletotrichum truncatum	Sordariomycetes	Ascomycota	90	150	59	72	371
Colletotrichum camelliae	Sordariomycetes	Ascomycota	90	139	65	77	371
Colletotrichum sp. *COLG25*	Sordariomycetes	Ascomycota	90	139	63	76	368
Colletotrichum karsti	Sordariomycetes	Ascomycota	90	139	57	71	357
Colletotrichum tropicale	Sordariomycetes	Ascomycota	89	139	63	77	368
Cadophora sp. *DSE1049*	Leotiomycetes	Ascomycota	105	138	75	91	409
Aspergillus latus	Eurotiomycetes	Ascomycota	95	137	53	58	343

Lange, L, Barrett, K, & Meyer, AS, 2021: *New Method for Identifying Fungal Kingdom Enzyme Hotspots from Genome Sequences. J. Fungi 7, 207*

Mycena citricolor, (Agaricales, Basidiomycota)
-a Hot Spot for pectin degrading CAZymes

Mycena citricolor **attack on coffee plants, leaves and fruits, illustrates massive degradation of plant tissue, creating holes in the leaves and sunking in parts of the berries**

Photo, courtecy of Andrew Dominick, University of Wisconsin-La Crosse

Conclusions

- Combining **CUPP, EPR & HotSpot** =>function-targeted enzyme-profiling & discovery
- Digestive enzyme secretome is an integrated part of speciation
  - *Secretome composition congruent with separation into Sections of Aspergillus & Penicillium*
- Ranking of enzyme capacity: similarities between cellulose- & xylan-active enzymes
- Diversity of enzyme function specificity richest for pectin-active enzymes
- Horizontal Gene Transfer appears to be frequent among aquatic, zoosporic fungi
- No AA enzymes/LPMOs found in rumen fungi. No lignin-active?
- EPR of digestive secretomes can give new input to analysis of complex genera

- Applied potential:
  - **”Hotspot” opens for optimized conversion blends xylan, pectin and lignin**
  - **AA3´s of unknown functions, prominent in digestive secretome composition!**

Next steps,
Optimizing development of Higher Value biobased products

Expand CUPP, ”F;F”, EPR, Hot Spot (and soon also blend composition):

to cover a broader spectrum of enzymes:

- AA´s, Esterases, Transferases, and Lyases
- Use all this in synergy with the new Alpha fold 3D finder
  - –a fantastic break through – a paradigm shift!

Expand functional annotation to cover also:

- Proteases and Lipases
- For this improved enzyme curation is needed

Notably: Strengthening EU Research Support efforts are needed! -JGI, BGI

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Closing remark:

”Circular & Bio-based food”, (from residues & side-streams etc) represents a huge opportunity for *Food security & Climate, Nutrition & Health, Biodiversity & Jobs!*

For this we need many new and improved enzymes:
Enzyme discovery for improved enzyme processing is KEY for valorizing the huge amount of biomass going wasted or downgraded

Thanks a lot for your attention

Lene