

Improved cellulosomes to enhance saccharification of industrially-suitable lignocellulosic biomass residues

ERA IB

Project acronym: ***FiberFuel***

Project no: ***EIB. 12.022***

Coordinator: ***Mariano Carrión-Vázquez***

ERA-IB-2 final conference, Berlin, 16./17.02.2016

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1. Project partners



CONSORTIUM (General Assembly)

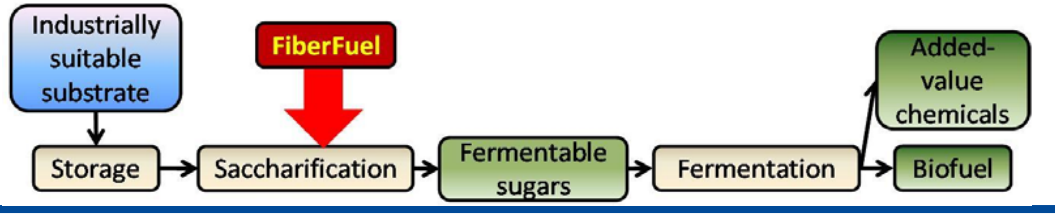
Partner #	Institution (legal name)	Type of Institution	IP	Country
1 (coordinator)	Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC)	National research council	Mariano Carrión-Vázquez	ES
2	Weizmann Institute of Science	University & research institute (SME)	Edward A. Bayer (Subcontracted: Alon Karpol. Designer Energy LTD)	IL (IL)
3	Centre National de la Recherche Scientifique (CNRS)	National research council	Mirjam Czjzek	FR
4	Instytut Fizyki Polskiej Akademii Nauk (IFPAN)	National academy of sciences (University)	Marek Cieplak (Subcontracted: Damien Thompson. University of Limerick)	PL (IE)
5	Abengoa Bioenergía Nuevas Tecnologías SA (ABNT)	Large industrial company (End user)	Carlos Blázquez	ES
(6)	Ludwig Maximilians Universität München	University	Hermann E. Gaub (external collaborator)	DE

Cross-disciplinary consortium involves biochemistry/molecular biology, bio-nanotechnology, structural biology, lab-on-a-chip and modelling.

- *Total project budget:* 640775€

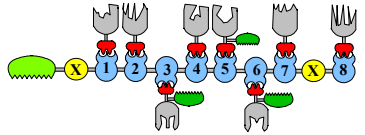
2. *FiberFuel* in a nutshell

Introduction



- *General project approach (problem to be solved)*

FiberFuel targets the rational design of optimized **designer cellulosomes** (*DC*s: cellulolytic enzyme systems based on a scaffolding protein) to overcome the major bottleneck in biomass industrial processing, namely **saccharification** (the conversion of cellulosic biomass to fermentable sugars). The goal is to **improve the efficiency of the saccharafication process** from low-value raw biomass materials (all of them renewable, sustainable and inexpensive) to **produce industrial-value chemicals**. Our **cross-disciplinary** approach involves bio-nanotechnology, structural biology, lab-on-a-chip and modeling.



- *Specific objectives*

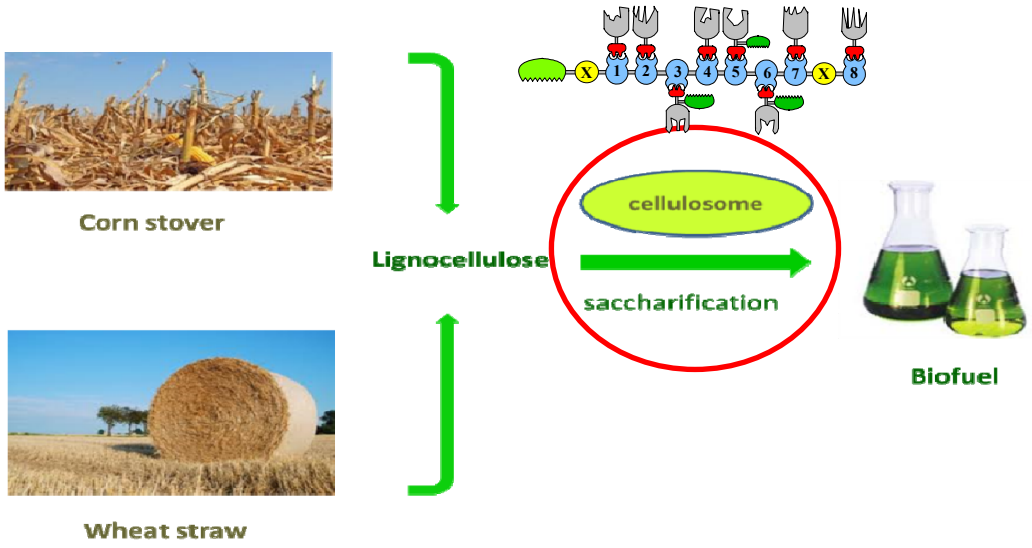
1. Characterization of natural cellulosomes (and non-cellulosomal cellulases) and candidate substrates.
This includes the **production** of substrates, enzymes and scaffolds, the **physico-chemical characterization** and the **improvement of thermal and mechanical stabilities**, the characterization of **atomic to supramolecular structure**, the development of an **standardized activity assay** to monitor enzymatic activity, and the **characterization of interactions** and its use for improvement of activity.

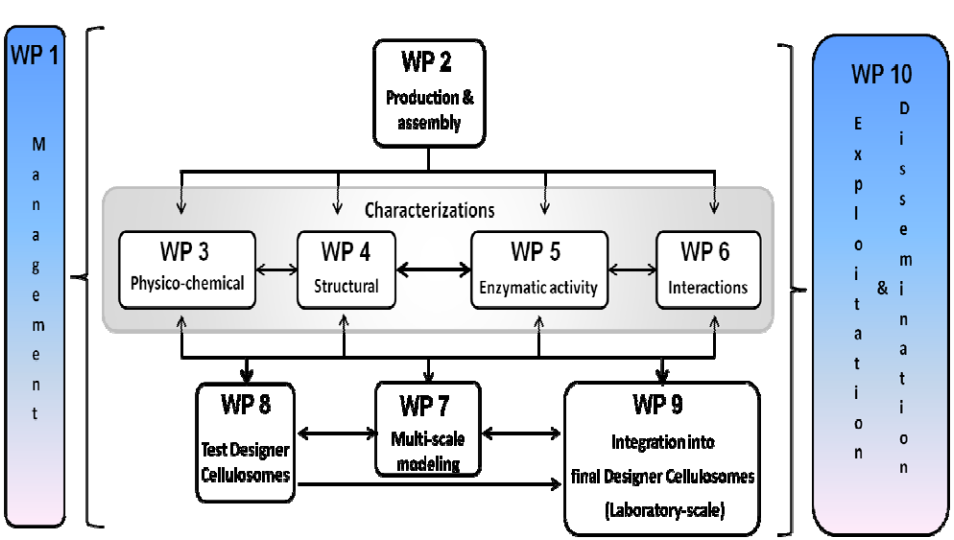
2. Multi-scale modeling of the cellulosome for *in silico* knowledge integration.
This will provide crucial support for the synthesis, assembly and characterization tasks, supplying detailed structural and energetic information to aid designing and interpreting experiments.

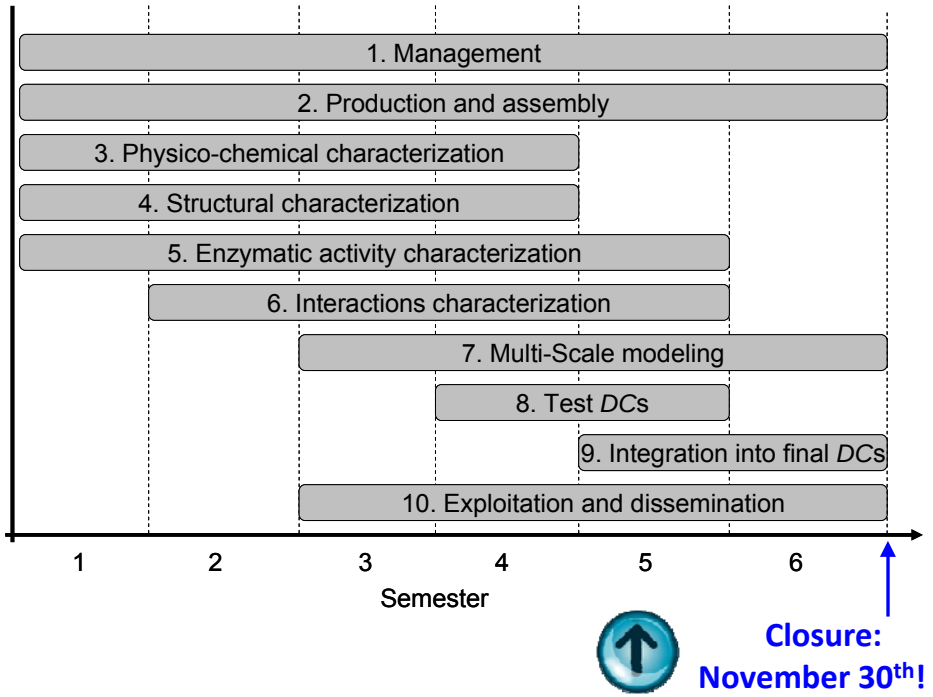
3. Rational design of optimized *DCs*.
The integration of the acquired knowledge from **1** and **2** and subsequent activity screening will allow constructing *DC*s optimized for degrading selected substrates, which will be validated at the **laboratory scale** and initial scaling up of the process to **pre-industrial** level.

Technical overview

WORK PLAN







Closure: November 30th!

EXPECTED RESULTS

1. A **standard activity assay** to monitor cellulosic substrate degradation.
2. **Test of the mechanical hypothesis** of the cellulosome.
3. **Supramolecular *DC* structure characterized** at the atomic and molecular levels.
4. **Molecular and supra-molecular models** of *DC*s.
5. **Optimized *DC*s obtained** based on rational design for the specific industrial substrates.

EXPECTED IMPACT

1. A new paradigm for the biotechnology field and market by developing a new product based on state-of-the-art technology with optimized cost/benefit and full legislative compliance.

2. Standardized technology to monitor catalytic activity by integrating research in both industrial plant-residues and biological catalysts.

3. High-value scientific products by integrating multidisciplinary capacities (nano-technology, molecular biology, biochemistry, chemistry, physics, biophysics and computer science) that includes technological ones in accordance with current specifications and design (industry requirements), and feedback from market research.

EXPLOITATION

1. *FiberFuel* is very likely to generate **new intellectual property** covering the optimized cellulosome derivatives (***DC*s**) and **processes for biomass degradation** into fermentable sugars. Also, the **standard activity assay** will be patented.

2. The ***DC*s** produced will be used and exploited by the industrial partner (ABNT). The developed technology will be **unique** and as such is not expected to conflict with other intellectual property.

3. Achievements

ACHIEVEMENTS: Milestones and Risk Plan

Milestone number	Milestone name	WP involved	Partner involved	Expected date	Means of verification
M1	Development of a standard activity assay to monitor cellulosic substrate degradation	5	DesEn, WEIZ & LMU	5th Semester	Assay ready to be used by the partners
M2	Testing the mechanical hypothesis of the cellulosomes	3	CSIC	3rd Semester	Convincing evidence of the relevance of the hypothesis
M3	Characterization of two industrial substrates	5	DesEn & WEIZ	2nd →6th Semester	Report on the characterization
M4	Characterization of physico-chemical properties of cellulosomes	3	WEIZ & CSIC	4th Semester	Report on physico-chemical properties
M5	Characterization of interactions of the cellulosome	6,7	LMU, CSIC, WEIZ, IFPAN & UL	5th Semester	Report on interactions
M6	Multi-scale modeling of DCs	7	IFPAN, TNI-UCC & CNRS	6th Semester	Report on the models
M7	Characterization of the supramolecular DC structure	4	CNRS, WEIZ & CSIC	4th Semester	Report on structures
M8	Constructing DCs based on rational design optimized for specific industrial substrates	8 & 9	CSIC, WEIZ, LMU, IFPAN,UL, CNRS, ABNT & DesEn	5-6th Semester	Report on the product
M9	Scaling up the process to pre-industrial level	9	ABNT	6th Semester	Report on scaling results
M10	Cost-benefit, and market analysis of the product and the process developed	9	ABNT	6th Semester	Report on market analysis results
M11	Life cycle of the product and the process developed	9	ABNT	6th Semester	Report on life cycle analysis results
M12	Results dissemination	10	CSIC, WEIZ, LMU, IFPAN,UL, CNRS, ABNT & DesEn	4-6th Semester	Publications

Done (4)
Ongoing (5)
Pending (3)

Risk Plan no.	Description	Associated Milestone no.
RP1	**a.** Develop a **relative** (to the ABNT standards) **enzymatic activity assay**, rather than an absolute assay **b**. We shall use a **combination of traditional enzymatic assays** that provide composite information for enzymatic activities of individual cellulosome components. **Novel high-throughput approaches** will also be developed during the course of the project	M1
RP2	If the mechanical hypothesis is proved true, we shall **construct *DC*s with high mechanostability** (or alternatively, we could use **architectures independent of mechanics**). If proven false, we would **disregard this variable**. Finally, if the results are inconclusive we would **stick to the mechanical hypothesis**	M2
RP3	We would **predict the supramolecular structures** of *DC*s using the structural information available	M7
RP4	A **trial-and-error approach** based on the knowledge generated by the consortium would be used	M8
RP5	If the optimized *DCs* were not cost-effective we would **produce the enzymes in the same microorganism that performs the fermentation**	M10

ACHIEVEMENTS: Deliverables

Deliverable no.	Deliverable name	WP no.	Partner involved	Nature	Dissemination level	Delivery date
D1	Protocol for standard activity assay	5	DesEn & WEIZ	R&D	Patent	5th Semester
D2	Results on the test for the mechanical hypothesis	3	CSIC	R	Publication & Patent	3rd Semester
D3	PDB files (from X-ray diffraction and SAXS) of the related structures	4	CNRS	R	Publication	4th Semester
D4	Interaction characterization	6,7	LMU, CSIC, WEIZ,IFPAN & UL	R	Publication	5th Semester
D5	Molecular and supra-molecular models of DCs	7	IFPAN, TNI-UCC & CNRS	R	Publication	6th Semester
D6	Optimized DCs	9	CSIC, WEIZ, LMU, IFPAN,UL, CNRS, ABNT & DesEn	R&D	Publication & Patent	5-6th Semester
D7	Report on substrates supply	2	ABNT	D	Internal report	2th(→6th) Semester
D8	System specifications for pre industrial level	9	ABNT	D	Internal report	6th Semester
D9	Market analysis of the product and the process defined	9	ABNT	D	Internal report	6th Semester
D10	Life cycle analysis	9	ABNT	D	Internal report	6th Semester

Done (4)
Ongoing (2)
Pending (4)

ACHIEVEMENTS: TASKS

WP1: Project management (CSIC): to coordinate the scientific & management aspects of the project. (Kick off Meeting: Madrid, January 16th, 2014)

WP2: Production of substrates and enzymes & assembly of *DCs* (WEIZ, CSIC, ABNT):

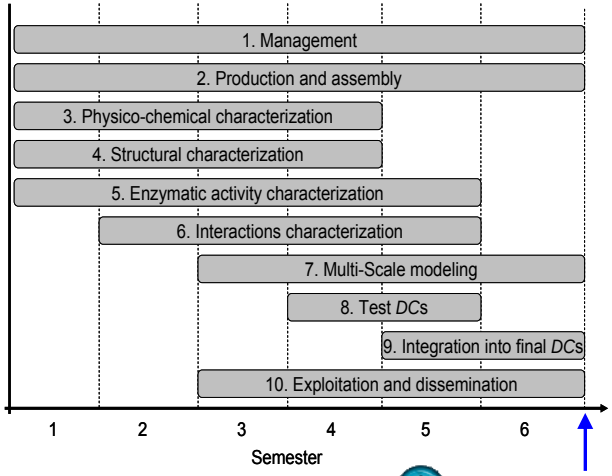
- **Task 2.1. Bioinformatics search for novel cellulosome components. WEIZ and CNRS.**
- **Task 2.2. Design and construction of mini-scaffoldins. WEIZ.**
- **Task 2.3. Design and conversion of enzymes to the cellulosomal mode. WEIZ.**
- **Task 2.4. Assembly and characterization of DCs. WEIZ**
- **Task 2.5. Preparation of fusion proteins for nanomechanical studies. CSIC**
- **Task 2.6. Production of substrates (wheat straw and corn stover) ABNT (crude biomass). (pre-treated biomass).**

WP3: Physico-chemical characterization and component improvement (CSIC, WEIZ)

- **Task 3.1. Thermal stability analysis. WEIZ.**
- **Task 3.2. *Improvement in cohesin-dockerin stability*. WEIZ.**
- **Task 3.3. Improvement in enzymatic stability.**
- **T ask 3.4. SMFS analysis. CSIC.**
- **Task 3.5. Cellulosomal improvement based on nanomechanics. CSIC.**

WP4: Structural characterization (CNRS)

- **Task 4.1. Detailed bioinformatic analysis of available structural data.**
- **Task 4.2. Crystallisation. CNRS.**
- **Task 4.3. *SAXS experiments*. CNRS.**



Closure: November 30th!

WP5: Substrate production and monitoring the enzymatic activity (DesEn, WEIZ, LMU) ABNT.

- **Task 5.1. Establishment of standardized set of low-throughput assays for analysis of cellulolytic activities. WEIZ and DesEn**
- **Task 5.2. *Establishing standardized low-throughput assays for analysis of hemicellulase activities.* DesEn.**
- **Task 5.3. Establishment of low-throughput assay systems for analysis of enzyme activities on natural cellulosic substrates. DesEn and WEIZ. ABNT.**
- **Task 5.4. *Establishment of standardized set of carbohydrate-binding assays*. DesEn and WEIZ**
- ***Task 5.5. Establishment of standardized set of medium- to high-throughput assay systems for analysis of enzymatic activities. WEIZ.***
- **Task 5.6. *High-throughput nanoassay for analysis of cellulase activity on recalcitrant cellulosic substrates.* WEIZ and LMU**

WP6: Characterization of interactions (LMU, CSIC, WEIZ):

- **Task 6.1. Establish basic set of interaction forces for the major components of a native cellulosome under standard conditions. LMU and CSIC.**
- **Task 6.2. *Establishing Molecular Force Balance with native modules*. LMU.**
- **Task 6.3. *Selection of suitable combinations of cohesin/dockerin variants*. LMU and WEIZ**

WP7: Multi-scale modeling (IFPAN, UL, CNRS, CSIC)

- **Task 7.1. *Atomistic MD: Self-assembly of multi-protein units*. TNI-UCC,IFPAN and LMU**
- **Task 7.2. *Atomistic MD: Binding specificity of cellulosome catalytic subunits*. TNI-UCC, fed back to CNRS, WEIZ and CSIC.**
- **Task 7.3. Atomistic MD: Mechanical properties of cellulosomal components. CSIC**
- **Task 7.4. *Coarse-Grain MD: Properties of component proteins*. TNI-UCC, IFPAN, fed back to LMU, WEIZ and CSIC.**
- **Task 7.5. *Coarse-Grain MD: Mechanical and thermal stability of extended architecture*. IFPAN**

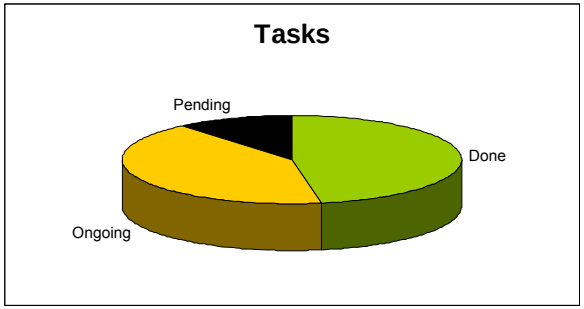
WP8: Designing test *DCs* (WEIZ, CSIC):

- **Task 8.1. Testing of new cellulosomal modules in the DCs context.**
- **Task 8.2. Testing of designer-cellulosome stability.**
- **Task 8.3. Implementation of novel DCs architectures.**
- ***Task 8.4. Test of DCs with optimized enzyme compositions.***
- ***Task 8.5. Testing of selected cellulosomes combined with free enzymes on various cellulosic substrates.***

WP9: Laboratory-scale level integration (CSIC, WEIZ, LMU, IFPAN, UL, CNRS, ABNT, DesEn):

- **Task 9.1. *Integration of all knowledge generated and industrial requirements into a set of DCs*.**
- ***Task 9.2. Performance test of the developed designs.***
- **Task 9.3. *Pre-industrial scaling up*. (100 ml flask and 1-5 l reactor) ABNT**

WP10: Exploitation and Dissemination (CSIC, WEIZ, LMU, IFPAN, UL, CNRS, ABNT, DesEn).



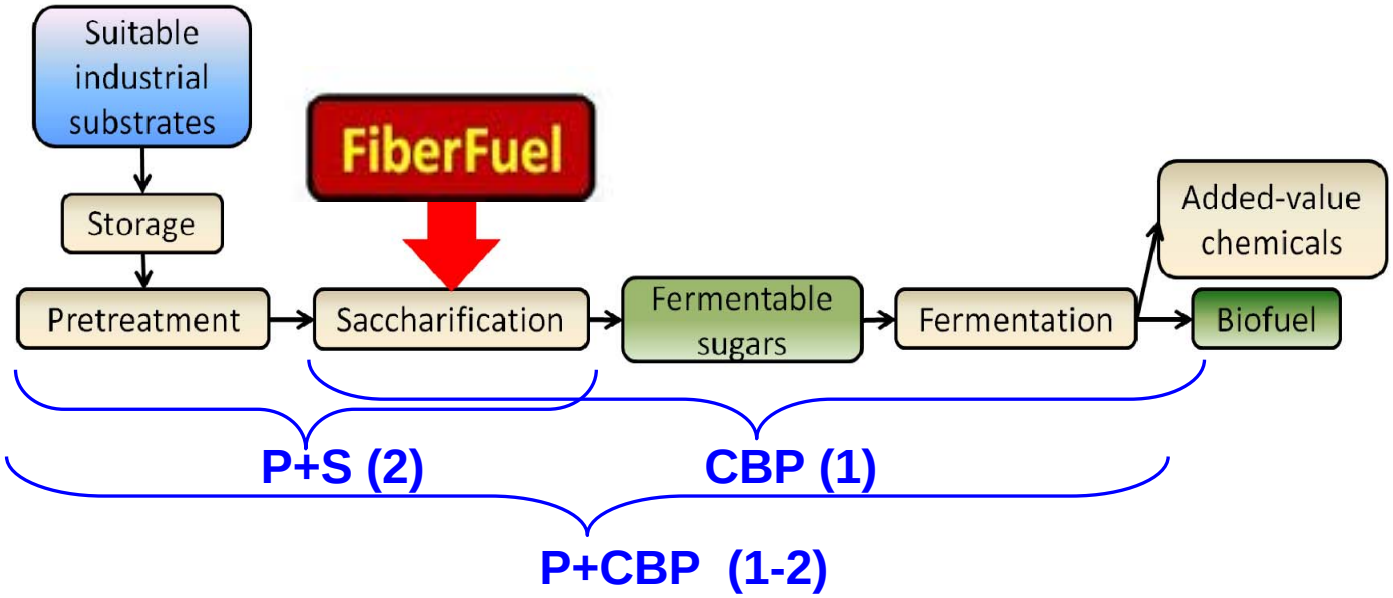
Done (17)
Ongoing (15)
Pending (4)

4. Plans for the future

NEW DEVELOPMENTS

1. We plan to collaborate with **Designer Energy** (a subcontractor of Weizmann with which we are already collaborating) towards the **integration of the Saccharification and Fermentation steps** towards the delopment of a **Consolidated Bioprocesing** (CBP) in *Clostridium thermocellum*

2. We also plan to collaborate with Profs. **Mercedes Ballesteros** & **José Miguel Oliva** (CIEMAT) towards the **integration of the Pretreatment and the CBP**.



Our design would be compatible with a mild Pre-treatment method based on **extrusion**, recently developed by Profs. Ballesteros and Oliva in the context another European Project.

-This method applies **presure and low temperatures** (65ºC).
- It would remain to integrate the Fermentation process (already initiated by DesEn in *Clostridium thermocellum*)

REQUESTED EU GRANTS (follow-up projects)

Using the acquired knowledge from this ERA-IB, we have submitted **2 european projects (H2020 & ERA-IB-2)** to **further improve the *DCs*** (by innovative and alternative approaches) and **integrating a CBP** (in *Clostridium thermocellum*) **and Pretratment**, for lignocellolosic residues

1. Proposal number: 720923-1
Work programme topic addressed: **BIOTEC-02-2016**
TOPIC : Bioconversion of non-agricultural waste into biomolecules for industrial applications
Title of Proposal: Consolidated bioprocessing of lignocellulosic bio-waste into bioproducts by improving designer cellulosomes, *Clostridium thermocellum* and pre-treatment.

Participant/IP	Legal Name	Country
1 Mariano Carrión-Vázquez (Coordinator)	AGENCIA ESTATAL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS, **CSIC**	SPAIN
2 Edward A. Bayer	WEIZMANN INSTITUTE OF SCIENCE, **WEIZ**	ISRAEL
3 Hermann E. Gaub	LUDWIG.MAXIMILIANS UNIVERSITAET MUENCHEN, **LMU**	GERMANY
4 Marek Cieplak	INSTYTUT FIZYKI POLSKIEJ AKADEMII NAUK, **IFPAN**	POLAND
5 Mirjam Czjzek	CENTRE NATIONAL DE LA RECHERCHE SCIENTIFIQUE, **CNRS**	FRANCE
6 Damien Thompson	UNIVERSITY OF LIMERCK, **ULIM**	IRELAND
7 Alon Karpol	DESIGNER ENERGY, **DE**	ISRAEL
8 Marta Tortajada	BIOPOLIS SL, **BIO**	SPAIN
9 Raúl Pérez	NanoGUNE, **nGUNE**	SPAIN
10 Mercedes Ballesteros	CENTRO DE INVESTIGACIONES ENERGÉTICAS, MEDIOAMBIENTALES Y TECNOLÓGICAS, **CIEMAT**	SPAIN
11 Caterina Coll	IMECAL S.A. , **IME**	SPAIN

2. Proposal ID: ERA-IB-16-057
Work programme topic addressed: 7TH Call **ERA-IB-2**
Project title: Improvement of designer cellulosomes, Clostridium thermocellum and pre-treatment towards a consolidated bioprocessing of lignocellulosic bio-waste into added-value bioproducts

Partner	Legal Name	Country
1 Dr Mariano Carrión Vázquez	AGENCIA ESTATAL CONSEJO SUPERIOR DE INVESTIGACIONES CIENTIFICAS **CSIC**	SPAIN
2 Prof Hermann Gaub	LUDWIG-MAXIMILIANS-UNIVERSITAET MUENCHEN **LMU**	GERMANY
3 Prof Marek Cieplak	INSTYTUT FIZYKI POLSKIEJ AKADEMII NAUK **IFPAN**	POLAND
4 Dr Jose Miguel Oliva	CENTRO DE INVESTIGACIONES ENERGETICAS, MEDIOAMBIENTALES Y TECNOLOGICAS-**CIEMAT**	SPAIN
5 Dr Maciej Mombrzynski	LUBELLA S.A. **LUBELLA**	POLAND

5. Project outcome

- *Show some visual outputs, charts, etc.*
- *Implementation and exploitation of results*

RESULTS

1. A standard activity assay to monitor cellulosic substrate degradation.
2. Test of the mechanical hypothesis of the cellulosome.
3. Supramolecular DC structure characterized at the atomic and molecular levels.
4. Molecular and supra-molecular models of DCs.
5. *Optimized DCs obtained based on rational design for the specific industrial substrates.*

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2. Standardized technology to monitor catalytic activity by integrating research in both industrial plant-residues and biological catalysts.

3. High-value scientific products by integrating multidisciplinary capacities (nano-technology, molecular biology, biochemistry, chemistry, physics, biophysics and computer science) that includes technological ones in accordance with current specifications and design (industry requirements), and feedback from market research.

EXPLOITATION

1. *FiberFuel* is very likely to generate **new intellectual property** covering the optimized cellulosome derivatives (***DC*s**) and **processes for biomass degradation** into fermentable sugars. Also, the **standard activity assay** will be patented.

2. The ***DC*s** produced will be used and exploited by the industrial partner (ABNT). The developed technology will be **unique** and as such is not expected to conflict with other intellectual property.

6. General Evaluation

- *Benefits of international collaboration; publications; exchange of researchers etc.*
- *Comments, feedback to ERA-IB*

Publications: 29 + 5

1.Artzi, L., Dassa, B., Borovok, I., Shamshoum, M., Lamed, R., and **Bayer, E. A.** (2014) Cellulosomics of the cellulolytic thermophile *Clostridium clariflavum. Biotechnol. Biofuels 7:100.*

2.Dassa, B., Borovok, I., Ruimy-Israeli, V., Lamed, R., Flint, H. J., Duncan, S., Henrissat, B., Coutinho, P., Morrison, M., Mosoni, P., Yeoman, C. J., White, B. A., and **Bayer, E. A.** (2014) Rumen cellulosomics: Divergent fiber-degrading strategies revealed by comparative genome-wide analysis of six ruminococcal strains. *PLoS ONE 9*, e99221.

3.Hamberg, Y., Ruimy-Israeli, V., Dassa, B., Barak, Y., Lamed, R., Cameron, K., Fontes, C. M., **Bayer, E. A.,** and Fried, D. B. (2014) Elaborate cellulosome architecture of *Acetivibrio cellulolyticus* revealed by selective screening of cohesin-dockerin interactions *PeerJ 2:e636*.

4.Artzi, L., Morag, E., Barak, Y., Lamed, R., and **Bayer, E. A.** (2015) *Clostridium clariflavum:* key cellulosome players are revealed by proteomic analysis. *mBio 6*, e00411-00415.

5.Cameron, K., Weinstein, J. Y., Zhivin, O., Bule, P., Fleishman, S. J., Alves, V. D., Gilbert, H. J., Ferreira, L. M. A., Fontes, C. M. G. A., **Bayer, E. A.**, and Najmudin, S. (2015) Combined crystal structure of a type-I cohesin: mutation and affinity-binding studies reveal structural determinants of cohesin-dockerin specificity. *J. Biol. Chem. 290*, 16215-16225.

6.Dassa, B., Uttukar, S. M., Klingeman, D. M., Hurt, R. A., Keller, M., Xu, J., Harish Kumar Reddy, Y., Borovok, I., Rozman Grinberg, I., Lamed, R., Zhivin, O., **Bayer, E. A**., and Brown, S. D. (2015) Near-complete genome sequence of the cellulolytic bacterium *Bacteroides (Pseudobacteroides) cellulosolvens* ATCC 35603. *Genome Announcements 3*, e01022-01015.

7.Rozman Grinberg, I., Yin, G., Borovok, I., Berg Miller, M. E., Yeoman, C. J., Dassa, B., Yu, Z., Mizrahi, I., Flint, H. J., **Bayer, E. A.**, White, B. A., and Lamed, R. (2015) Functional phylotyping approach for assessing intraspecific diversity of *Ruminococcus albus* within the rumen microbiome. *FEMS Microbiol. Lett. 362*, 1-10.

8.Schoeler, C., Malinowska, K. H., Bernardi, R. C., Durner, E., Ott, W., **Bayer, E. A**., Schulten, K., Nash, M. A., and **Gaub, H. E.** (2015) Mapping mechanical force propagation through biomolecular complexes. *Nano Letters 15*, 7370-7376.

9.Slutzki, M., Reshef, D., Barak, Y., Haimovitz, R., Rotem-Bamberger, S., Lamed, R., **Bayer, E. A.**, and Schueler-Furman, O. (2015) Crucial roles of single residues in binding affinity, specificity and promiscuity in the cellulosomal cohesin-dockerin interface. *J. Biol. Chem. 290*, 13654-13666.

10. Stern, J., Kahn, A., Vazana, Y., Shamshoum, M., Moraïs, S., Lamed, R., and **Bayer, E. A.** (2015) Significance of relative position of cellulases in designer cellulosomes for optimized cellulolysis. *PLoS ONE 10:e0127326*.

11.Voronov-Goldman, M., Yaniv, O., Gul, O., Yoffe, H., Salama-Alber, O., Slutzki, M., Levy-Assaraf, M., Jindou, S., Shimon, L. J. W., Borovok, I., **Bayer, E. A**., Lamed, R., and Frolow, F. (2015) Standalone cohesin as a molecular shuttle in cellulosome assembly. *FEBS Lett. 589*, 1569-1576.

12.Gunnoo, M., Cazada, P.-A., Galera-Prat, A., Nash, M. A., **Czjzek, M.**, **Cieplak, M.**, Alvarez, B., Aguilar, M., Karpol, A., **Gaub, H. E.**, **Carrion-Vazquez, M.**, **Bayer, E. A.**, and Thompson, D. (2016) Nano-scale engineering of designer cellulosomes. *Adv. Mater.* DOI: 10.1002/adma.201503948.

13. Malinowska, K., Verdorfer, T., Meinhold, A., Milles, L.F., Funk, V., **Gaub, H.E**., and Nash, M. A.* Redox-Initiated Hydrogel System for Detection and Real-Time Imaging of Cellulolytic Enzyme Activity. ChemSusChem 7(10): 2825-2831, 2014.

14.Jobst, M. A., Schoeler, C., Malinowska, K., and **Nash, M. A**.*Investigating Receptor-Ligand Systems of the Cellulosome using AFM-based Single-molecule Force Spectroscopy. Journal of Visualized Experiments, 82, e50950, 2013.

15.Malinowska, K.H., Rind, T., Verdorfer, T., **Gaub, H.E.**, and Nash, M.A.*Quantifying Synergy, Thermostability, and Targeting of Cellulolytic Enzymes and Cellulosomes with Polymerization-Based Amplification.Analytical Chemistry, 87(14):7133-7140, 2015.

16. Jobst, M., Milles, L., Schoeler, C., Ott, W., Fried, D. B., **Bayer, E. A**., **Gaub, H. E**., and Nash, M. A.* Resolving Dual Binding Conformations of Cellulosome Cohesin-Dockerin Complexes using Single-Molecule Force Spectroscopy.eLife, DOI: 10.7554/eLife.10319, 2016.

17. Schoeler, C., Bernardi, R. C., Malinowska, K. M., Durner, E., Ott, W., **Bayer, E. A**., Schulten, K., Nash, M. A.*, and **Gaub, H.E.**Mapping Mechanical Force Propagation Through Biomolecular Complexes.Nano Letters, 15(11): 7370-7376, 2015.

Publications (Cont.)

18.M. Chwastyk, A. Galera, M. Sikora, A. Gomez-Sicilia, **M. Carrion-Vazquez** and **M. Cieplak**, Theoretical tests of the mechanical protection strategy in protein nanomechanics, Proteins: Struct., Funct., Bioinf. 82, 717-726 (2014)

19.M. Chwastyk, M. Jaskólski and **M. Cieplak**, Structure-based thermodynamic and mechanical stability of plant PR-10 proteins with cavities, FEBS J. 281, 416-429 (2014)

20. B. RóŻycki, Ł. Mioduszewski and **M. Cieplak**, Unbinding and unfolding of adhesion protein complexes through stretching: interplay between shear and tensile mechanical clamps, Proteins: Structure, Function, and Bioinformatics, 82, 3144-3153 (2014)

21. M. Chwastyk and **M. Cieplak**, Knotted proteins under tension, Israel J. Chem. 54, 1241-1249 (2014)

22. B. RóŻycki and **M. Cieplak**, Citrate synthase proteins in extremophilic organisms – studies within a structure-based model, J. Chem. Phys. 141, 235102 (2014)

23. M. Wojciechowski, D. Thompson, and **M. Cieplak,** Mechanostability of cohesin-dockerin complexes in a structure-based model: Anisotropy and lack of universality in the force profiles, J. Chem. Phys. 141, 245103 (2014)

24. M. Chwastyk, A. B. Poma, and **M. Cieplak**, Statistical radii associated with amino acids to determine the contact map: fixing the structure of a type I cohesin domain in the *Clostridium thermocellum* cellulosome, Phys. Biol. 12, 046002 (2015)

25. M. Chwastyk and **M. Cieplak**, Cotranslational folding of deeply knotted proteins, J. Phys.: Cond. Matter 27, 354105 (2015)

26. B. RóŻycki, **M. Cieplak**, and **M. Czjzek**, Large conformational fluctuations of the multi-domain Xylanase Z of *Clostridium thermocellum,* J. Struct. Biol. 191, 68-75 (2015)

27. A. B. Poma, M. Chwastyk, and **M. Cieplak**, Polysaccharide-protein complexes in a coarse-grained model, J. Chem. Phys. B 119, 12028-12041 (2015)

28. K. Wołek, A. Gomez-Sicilia, and **M. Cieplak**, Determination of contact maps in proteins: a combination of structural and chemical approaches, J. Chem. Phys. 143, 243105 (2015)

29. G.Nawrocki, P. –A. Cazade, D.Thompson, and **M. Cieplak**, Peptide recognition capabilities of cellulose in molecular dynamics simulations, J. Phys. Chem. C 119, 24402-24416 (2015)

Unpublished articles

30. A. B. Poma, M. Chwastyk, and **M. Cieplak**, Coarse-grained model of the native cellulose Iα and the transformation pathways to the Iβ allomorph (submitted to Cellulose)

31. M. Chwastyk, D. Thompson, and M. Cieplak, Designer cohesins - the influence of mutations on the mechanical and thermodynamic stability of proteins (manuscript)

32. B. RóŻycki and M. Cieplak, Stiffness of the C-terminal disordered linker affects the geometry of the active site in endoglucanase Cel8A (manuscript)

33. Galera-Prat, A. Shamshoum, M., Barak, Y., **Bayer,E.** & **Carrión-Vázquez, M**. Testing the mechanical hypothesis of the cellulosome (manuscript) .

34. Vera, A.M. & Carrión-Vázquez, M. Direct identification of protein-protein intermolecular forces in protein nanomechanics by AFM (manuscript).

PhD Theses (defended): 5

-Albert Galera Prat. Nanomechanics of the cellulosome: implications for the activity of the system.Universidad Autónoma de Madrid. 2016 (February 5th, 2016).

- Andrés Manuel Vera Gómez. Unequivocal nanomechanics of intermolecular interactions in proteins. *Universidad de Sevilla. (May 22nd, 2015).*

- *Grzegorz Nawrocki.* Interactions of biomolecules with solids in molecular dynamics simulations. Institute of Physics, Polish Academy of Sciences. 2015.

-*Mateusz Chwastyk* – Dynamics of proteins with knots, cavities, and scaffolds. Institute of Physics, Polish Academy of Sciences. To be defended in 2016

-Johanna Stern, Contribution of modular architectural configuration and enzyme composition on designer cellulosome action, Weizmann Institute of Science, February 2016.

Researchers interchange: 2

- Grzegorz Nawrocki, 3 months. Visit from IFPAN to ULIM.
- Mateusz Chwastyk, 3.5 months. Visit from IFPAN to ULIM

Invited lectures at international conferences: 34

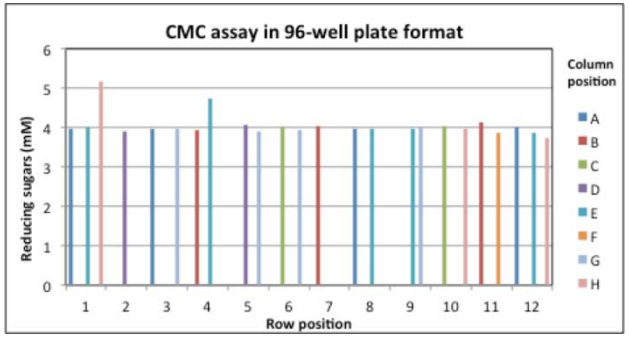
Summary of benefits

This grant has been extremely useful for promoting collaborative activities in Europe through the **creation of a new consortium**. Thanks to it, the **basic objectives** of these consortium have been or will be soon achieved and new synergies have appeared . Most importantly, we have envisioned **new projects to further develop the biorefinery concept** in a more integrated and efficient way.

7. Specific results by partner

Partner 2 – Ed Bayer

Characterization of enzyme systems and candidate substrates



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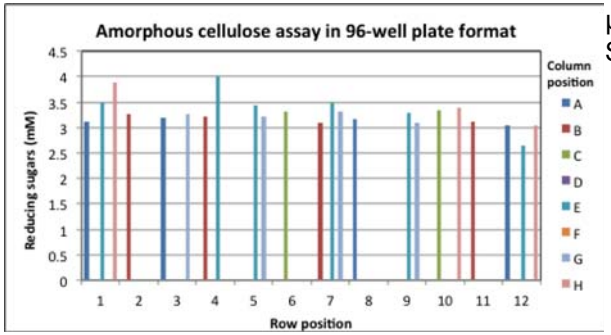
SD: 0.29



μ: 3.2 mM
SD: 0.27

Objective 1

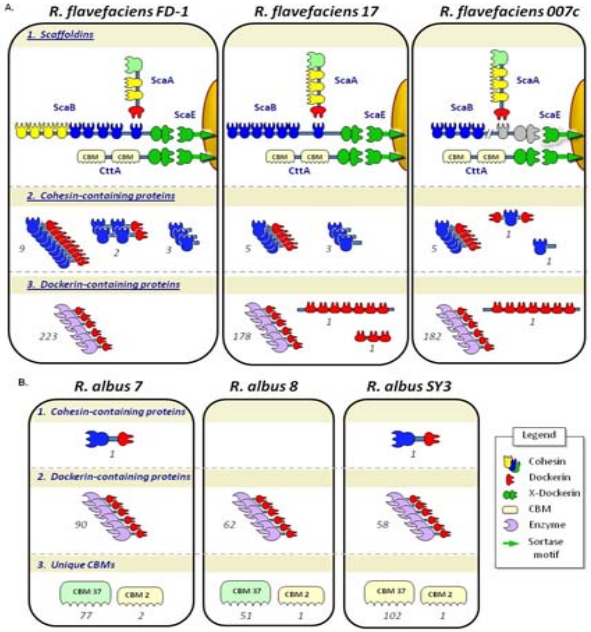
WP5, Tasks 5.1, 5.5

Milestone M1

Deliverable D1

Bioinformatics-based characterization of natural cellulosomes

Scaffoldins

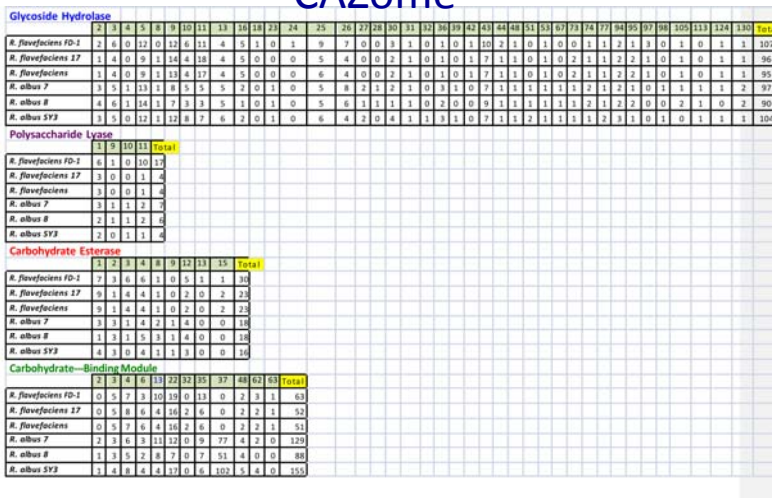


Objective 1 **WP2, Task 2.1**

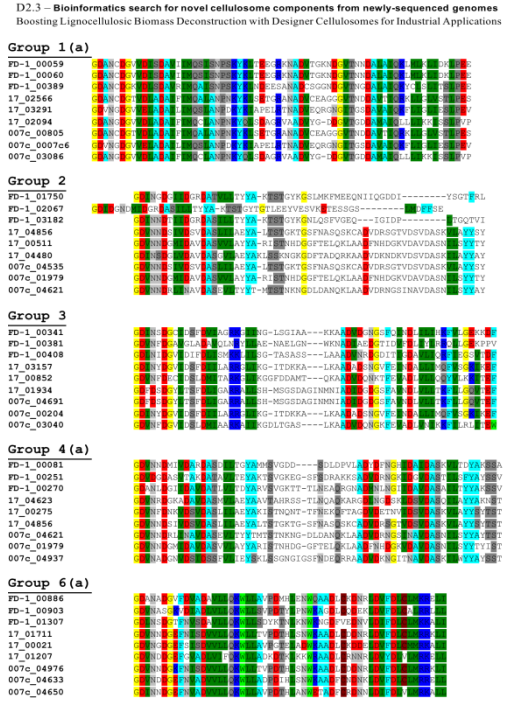
Typical cellulosome components

Cellulosome-producing bacteria	Cohesin-containing proteins	Cohesin modules	Dockerin-containing proteins	CAZymes			Multifunctional proteins		CBMs	
				GHs	PLs	CEs	Total	Novel[a]	Total	CBM37
R. flavefaciens FD-1	17	27	223	107	17	30	23	14	63	-
R. flavefaciens 17	11	21	180	96	4	23	19	2	52	-
R. flavefaciens 007c	10	16	183	95	4	23	17	-	51	-
R. albus 7	1	1	90	97	7	18	8	-	129	77
R. albus 8	-	-	62	90	6	18	4	4	88	51
R. albus SY3	1	1	58	104	4	16	7	1	155	102

CAZome

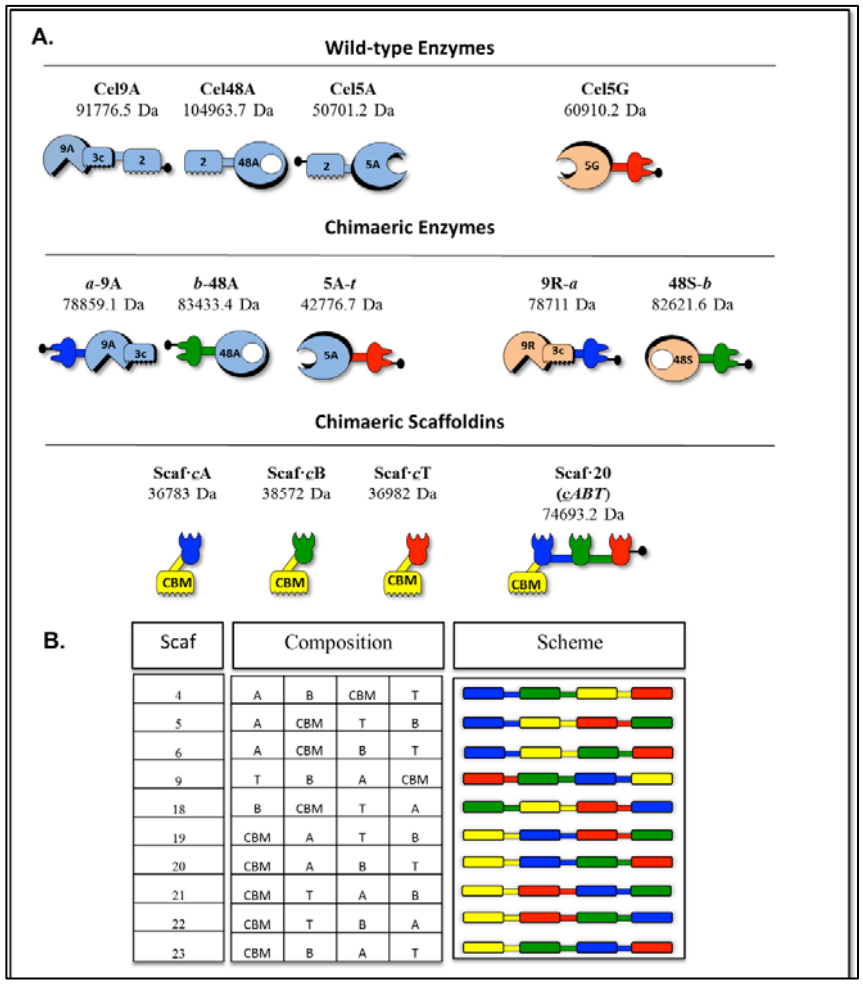


Dockerins



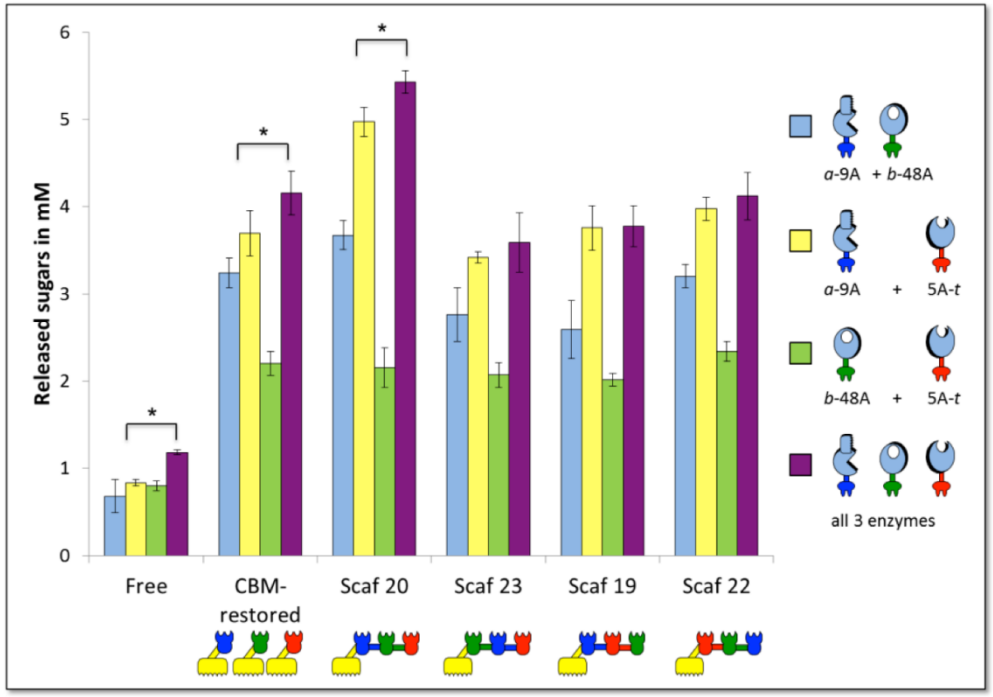
Rational design of *DCs*

Schematic representation of recombinant proteins used



Objective 3 WP2, Tasks 2.2, 2.3, 2.4
Milestone M8 Deliverable D6

Synergistic activity of the combinations of the recombinant enzymes.



Objective 3 WP2, Tasks 8.1, 8.3, 8.4 Milestone M1

Supply of Cellulosome Components to Partners

Protein modules (cohesins, dockerins, etc) and plasmids have been sent so far to Partners 1 and 8

Objective 1 WP3, Tasks 3.4, 3.5 WP6, Tasks 6.1, 6.2, 6.3
Milestones M2, M4, M5 Deliverables D2, D4

Technical overview

WP2: Production of substrates and enzymes, and assembly of Designer Cellulosomes:

To identify, design, optimize, produce and assemble the components for their development

Task 2.1. *Bioinformatics search for novel cellulosome components.*

Genomes of cellulosome-producing bacteria were sequenced and analyzed for cellulosomal components. These include cohesin, dockerin, CBMs, and enzymes -- glycoside hydrolases (GHs)

Bacterial "cellulosomics" analyzed: *Acetivibrio cellulolyticus, Bacteroides cellulosolvens, Clostridium clariflavum, Clostridium thermocellum* (various strains), *Ruminococcus flavefaciens* (3 strains) and *Ruminococcus albus* (3 strains).

The analysis provided novel components for construction of designer cellulosomes.

Task 2.2. *Design and construction of mini-scaffoldins.*

Establishment of cohesin library.

Establishment of scaffoldin library.

Adaptor scaffoldins: Design and construction of highly structured designer cellulosomes for increased integration of enzymes.

Technical overview

WP2: Production of substrates and enzymes, and assembly of Designer Cellulosomes:

Task 2.3. *Design and conversion of enzymes to the cellulosomal mode.*

Establishment of dockerin library.

Establishment of enzyme library.

Analysis of enzyme action.

Production of thermostable enzymes by directed evolution, consensus-guided mutagenesis, computational methods and site-directed mutagenesis.

Production of dockerin-containing enzymes.

Task 2.4. *Assembly and characterization of DCs.*

A standardized set of low- and medium-throughput assays was established for analysis of cellulolytic activities

Insight into the significance of relative location of cellulases in designer cellulosomes for optimized cellulolysis was achieved.

Synthesis of a library of designer cellulosomes and their analysis

WP 5: Substrate production and monitoring the enzymatic activity

Establishment of standardized set of low-throughput assays for analysis of cellulolytic activities

main results:

Applying enzymatic assay on set of Clostridium thermocellum enzymes, and rapidly determining the mode of action

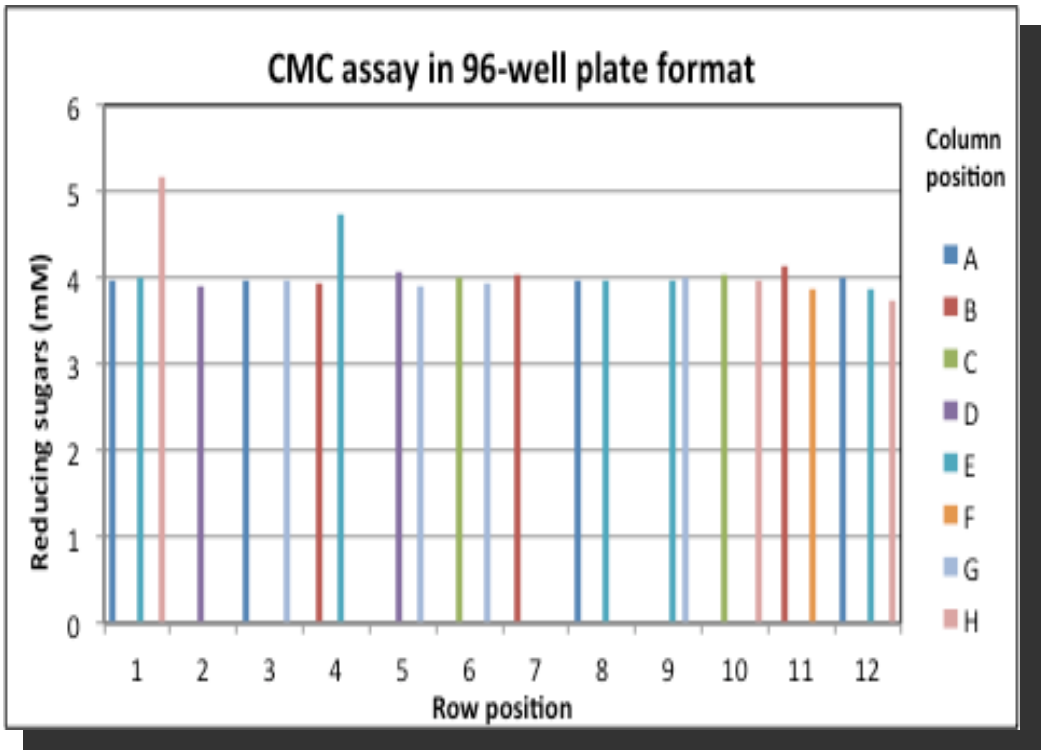
enzyme	CMCase activity	Amorphus cellulose activity	Ratio CMC/amorphus	literature
CelD	2848	37	77	endoglucanase
CelA (mutant)	1635	22	72	endoglucanase
Cel E	574	26	21	endoglucanase
CelI	360	46	7.7	Proccesive-endo
Cel R	136	17	8	Proccesive-endo
Cel k	0.85	51	0.01	Exoglucanase
cbhA	5	31	0.15	Exoglucanase

The assay developed in WP5.I can discriminate between the endoglucanses and exoglucanases from the CMC/amorphous cellulose activity ratio (>5: Endo; <1:Exo)

WP 5: Substrate production and monitoring the enzymatic activity

Establishment of standardized set of medium- to high-throughput assay systems for analysis of enzymatic activities

main results:



The assay developed in WP5.4 can detect the activity of endoglucanses and exoglucanases quite homogeneously in HTS of 96 wells format.

Partner 1 – Mariano Carrión-Vázquez

(Confidential results)

Partner 6 – Herman Gaub (external collaborator)

Characterization of interactions

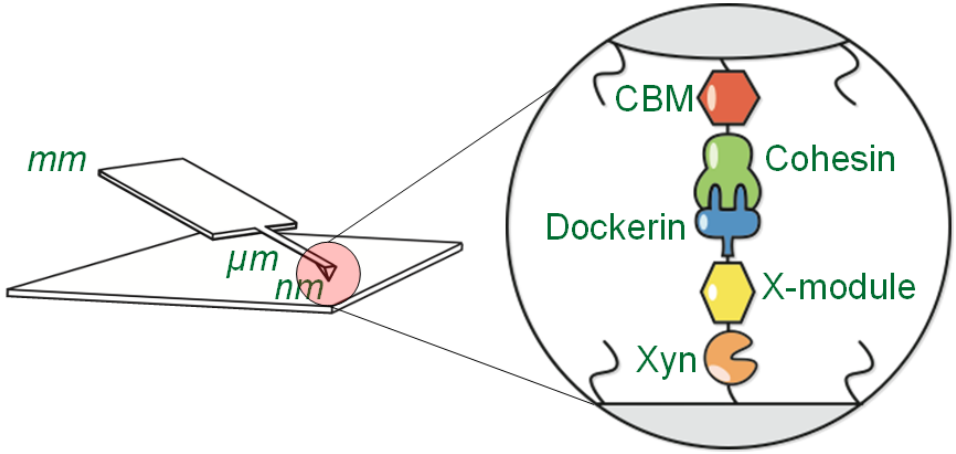
Coh-Doc binding pairs tested with SMFS:

- Ct.DocS (type I)
- Ct.CipA X-Doc (type II)
- Rf.Ctta X-Doc (type III)
- Rf.ScaA Doc (type III)

Single-molecule rupture forces (approx.)

- ~125 pN
- ~125 pN
- **500-750 pN (!)**
- ~125 pN

AFM force spectroscopy



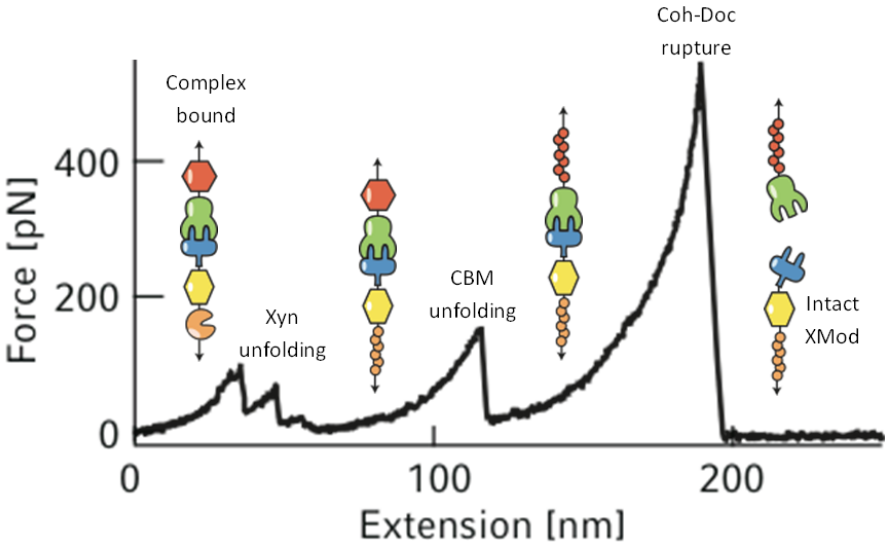
Objective 1

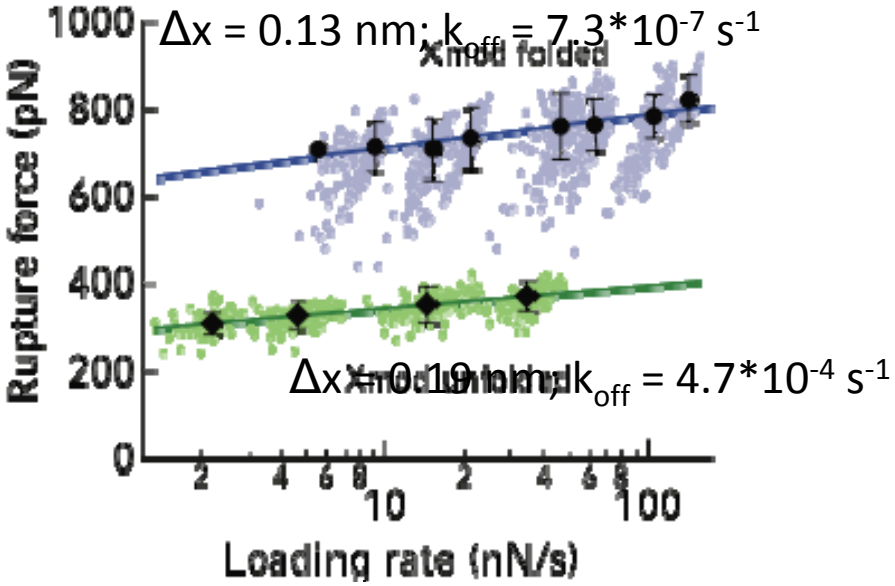
WP6, Task 6.1

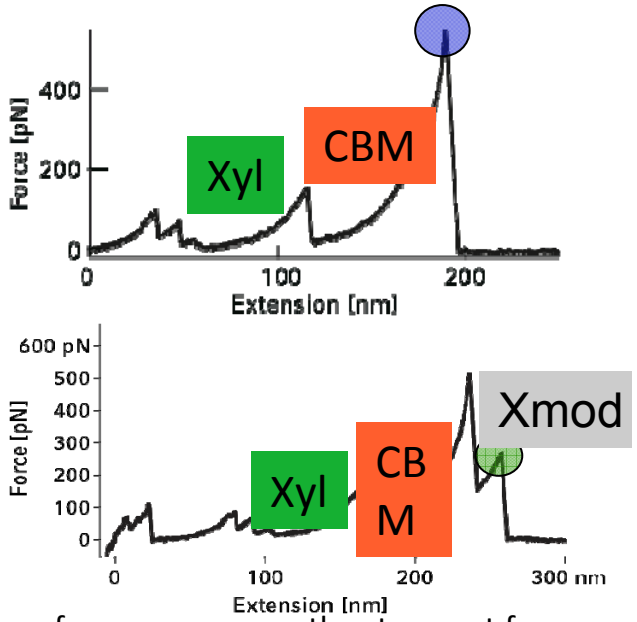
Milestone M5

Deliverable D4

Examples from *R. Flavefaciens* Ctta X-Doc (type III)







- Affinity of this complex is commonplace (K_D~ 20 nM), however, the rupture forces are among the strongest for a receptor-ligand complex. This suggests the complex is selected for high mechanical resistance.
- Extremely short Δx (0.13 nm) consistent with a stiff interaction zone. Upon unfolding of XMod, Δx inceases (0.19 nm). Suggests XMod is active in mechanical stabilization of the complex.

WP3: Report on Thermo/Mechano-stability



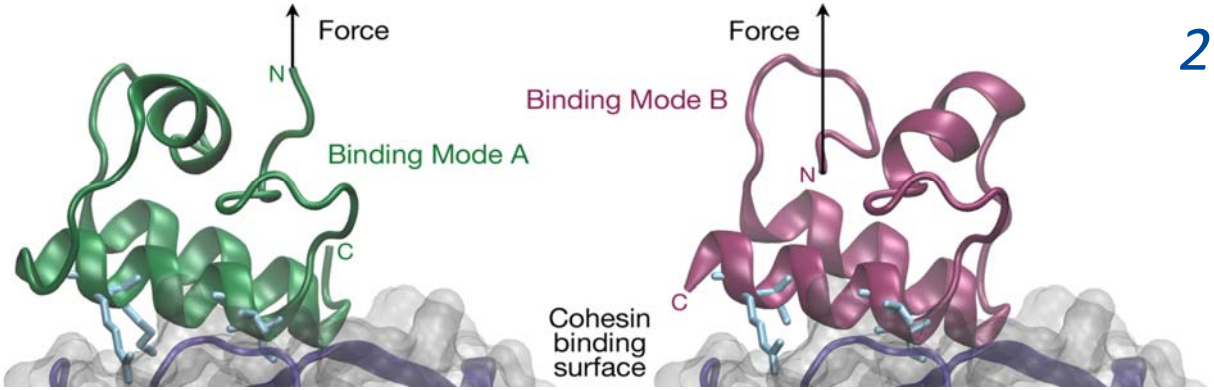
- *Proposed: Testing mechanical responses of designer cellulosomes using molecular force balance/AFM force assay*
- *Mechanical hypothesis has been substantiated. ~10 or more mechano-superstable cellulosome components have been verified.*
- *Understanding the relationship between molecular mechanics and catalytic performance.*

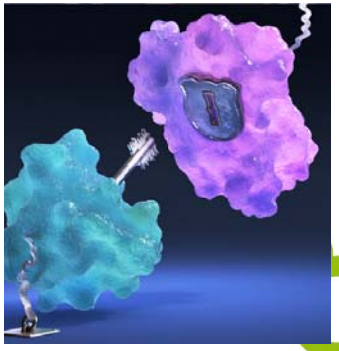
Unique mechanism of Coh-Doc recognition: dual binding modes



- *Hypothesis: Coh-Doc complexes assemble in two possible ways*
- *Implementation: Use SMFS to quantify binding mode populations*

Analogy: key fits lock in 2 different ways





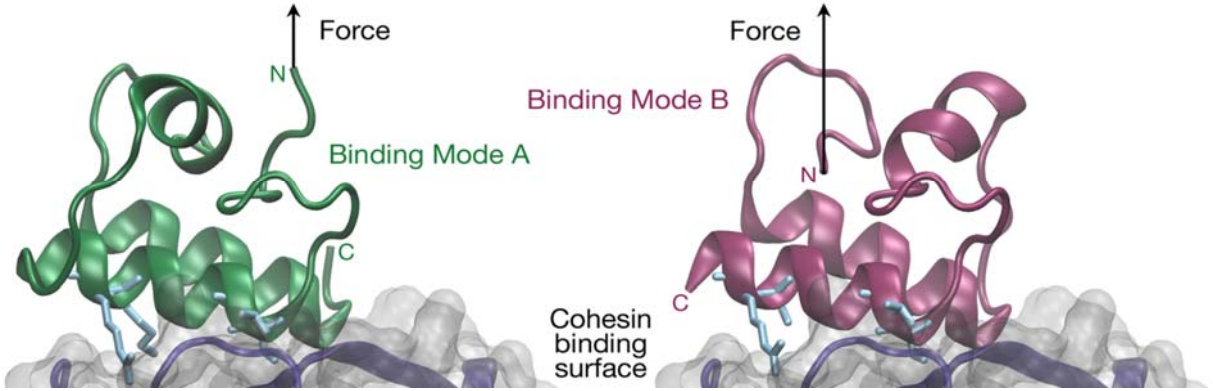
Jobst, M.A., Milles, L.M., et. al., and Nash, M.A.* **eLife** 10.7554/eLife.10319, 2015.

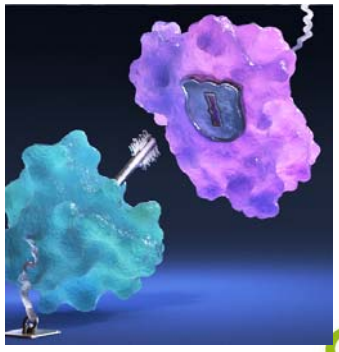
Unique mechanism of Coh-Doc recognition: dual binding modes



- *Hypothesis: Coh-Doc complexes assemble in two possible ways*
- *Implementation: Produce mutations to knock out binding modes. Use SMFS to quantify binding mode populations*

Analogy: key fits lock in 2 different ways

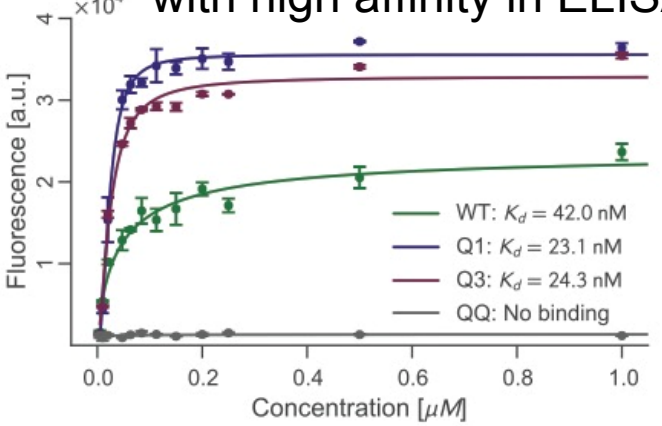




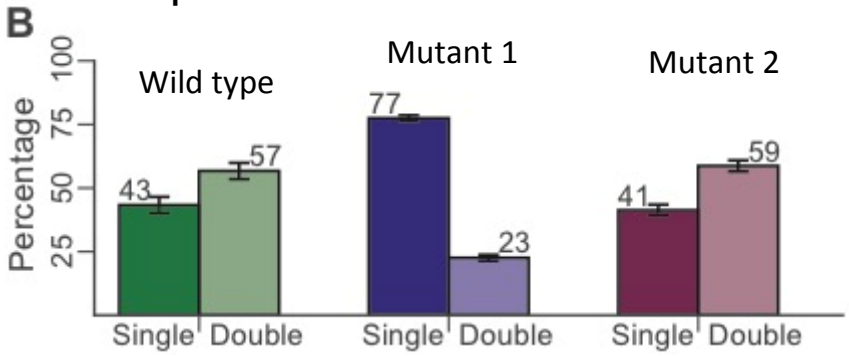
Jobst, M.A., Milles, L.M., et. al., and Nash, M.A.* **eLife** 10.7554/eLife.10319, 2015.

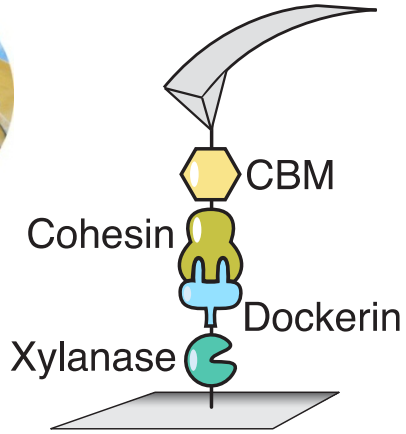
Coh-Doc recognition: dual binding mode

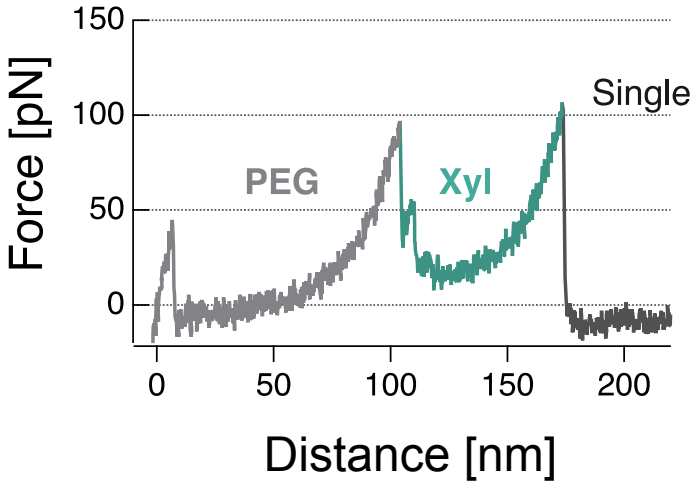
Both knockouts bind with high affinity in ELISA

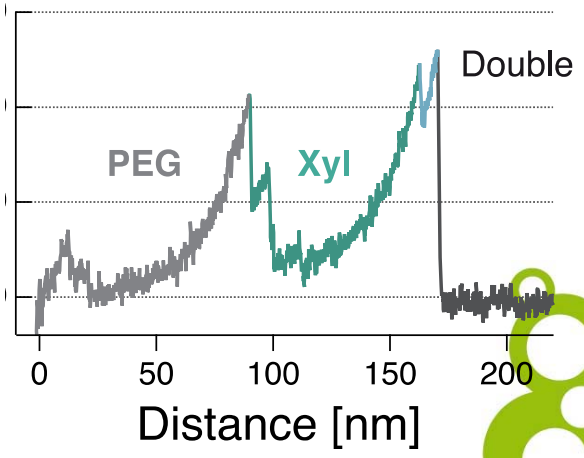


Single-molecule data show clear preferences for mutants







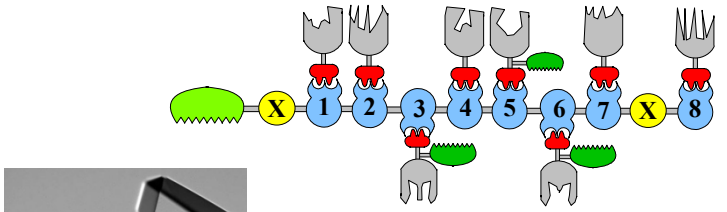


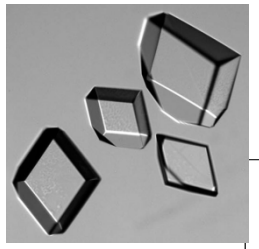
Partner 3 – Mirjam Czjzek

Understanding structure-function relationships of cellulosomal complexes

Technical Overview

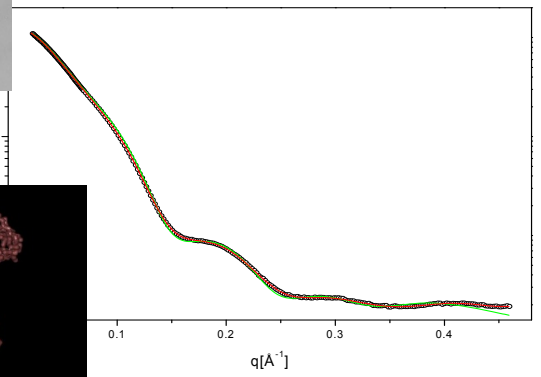
Crystal structure of modules and complexes



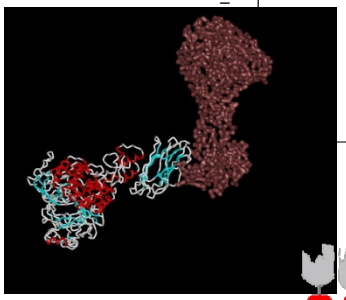


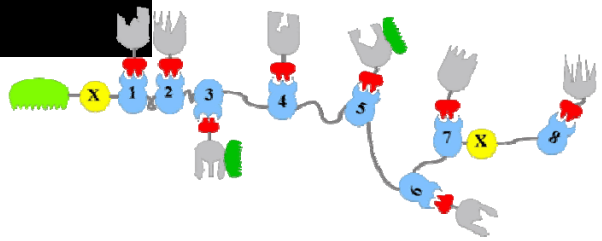
C. cellulolyticum

SAXS of complexes and full length proteins including their flexible linkers



Molecular modeling and biophysical analyses in collaboration with partners 1,2,4 and 6



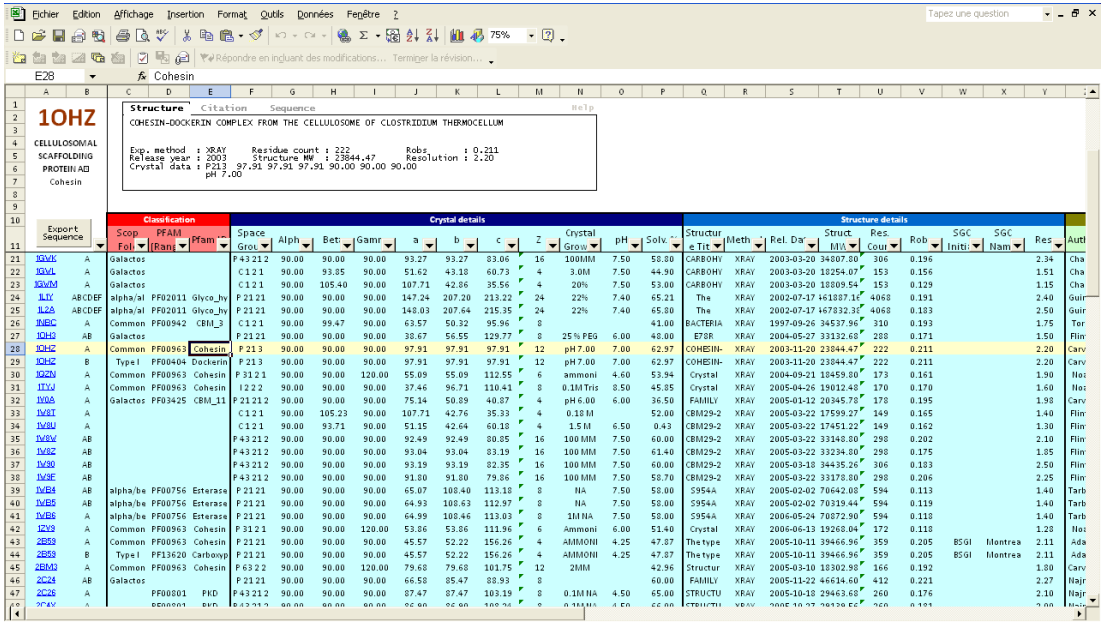


to understand the structural determinants of the synergy of cellulosomes

Creation of an interactive cellulosomal structure data base

With the aim of rationalizing the structural, biophysical and biochemical data on cellulosomal components, we are preparing an **interactive data base** that summarizes all this information for a given structure.

Those present in public data bases are aleady assembled (see figure). We will also include data from SAXS measurements, from kinetic studies and those produced during this project.



Objective 1

WP4, Tasks 4.1, 4.2 & 4.3

Milestone M7

Deliverable D3

Crystal structures

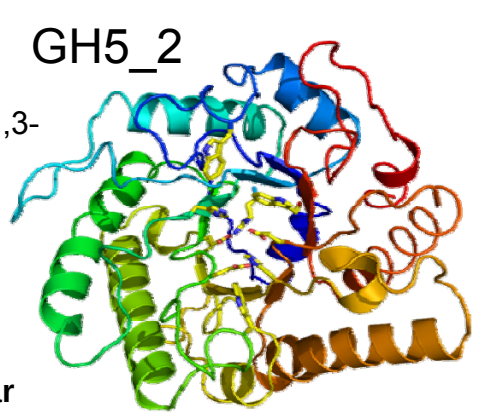
We have determined two new crystal structures of cellulases:

i) a **GH5 subfamily 2** of marine origin (*Z. galactanivorans*) with specificity for mixed linked β1,3-1,4 substrates.

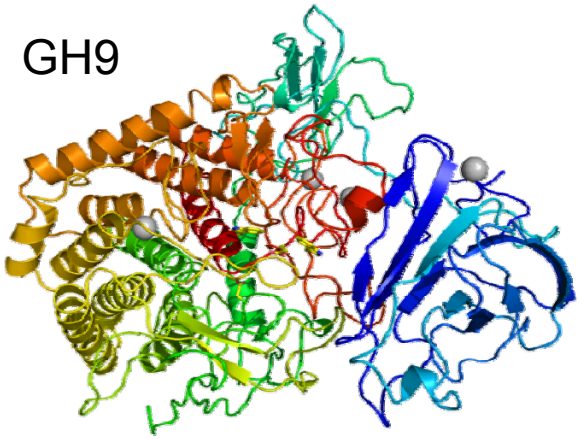
ii) **Cel9E full length** from *C. cellulolyticum.*

We also **provided models of full length cellulosomal components** with corresponding experimental SAXS data to **partner 6** for **molecular modelling and will provide more in near future.**

GH5_2



GH9

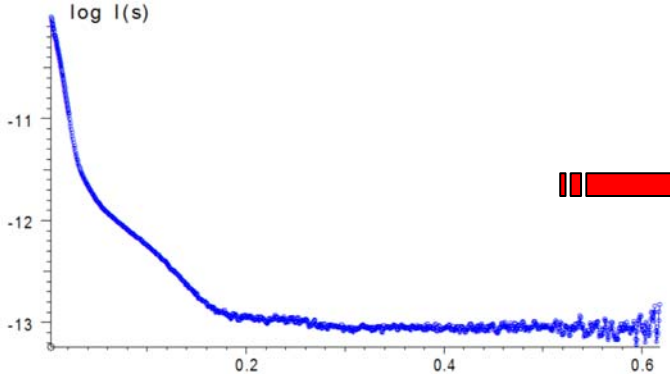


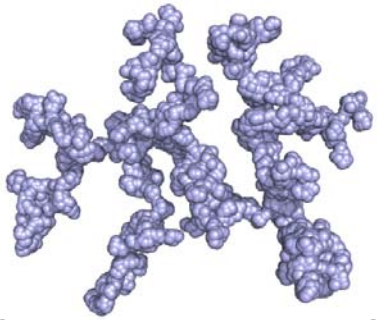
Summary



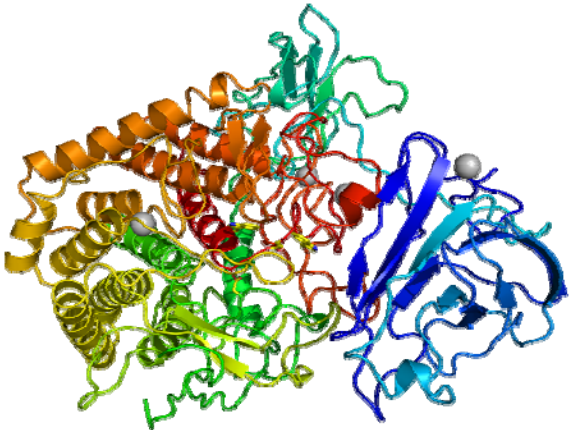
- *Crystal structures of (new) GH5 and (modular) GH9 enzymes*
- *SAXS curves from cellulosomal complexes*

- SAXS curves obtained for ScaA in complex with Cel9A
- SAXS curves obtained for CipA in complex with Cel8





CipA(withoutXD)-Cel8 envelope in solution



- *Basis for molecular modelling in the coming year - will help understand and interpret biophysical and enzymatic data*

Różycki B, Cieplak M, **Czjzek M**. (2015) Large conformational fluctuations of the multi-domain Xylanase Z of *Clostridium thermocellum*. *J. of Struct. Biol*., **191**(1):68-75

Partner 4 – Marek Cieplak

Single proteins: cohesins
1AOH 7.3, 7.4, 7.5
1G1K 7.3, 7.4, 7.5
1ANU 7.3, 7.4, 7.5

Complexes; cohesins-dockerins
4IU3 7.1, 7.4, 7.5
2B59 7.1, 7.4, 7.5
1OHZ 7.1, 7.4, 7.5

Hydrolases:
1RQ5 7.2, 7.3

Cellulose Bindind Modules
1NBC 7.2, 7.3, 7.5
3P6B 7.2, 7.3, 7.5

scaffoldins:
3KCP 7.4, 7.5
4B9F 7.4, 7.5
4FL4 7.4, 7.5

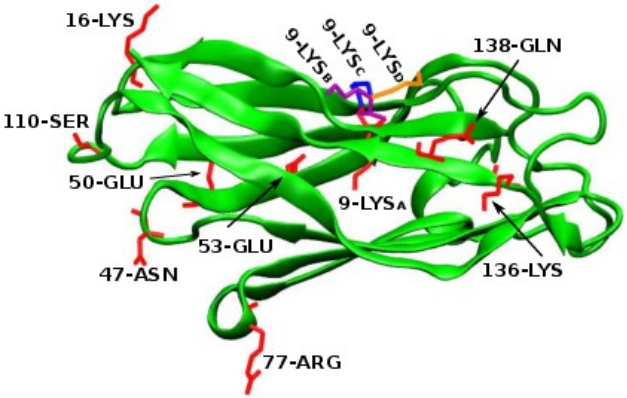
Objective 2

WP7, Tasks 7.1-7.5

Milestone M6

Deliverable D5

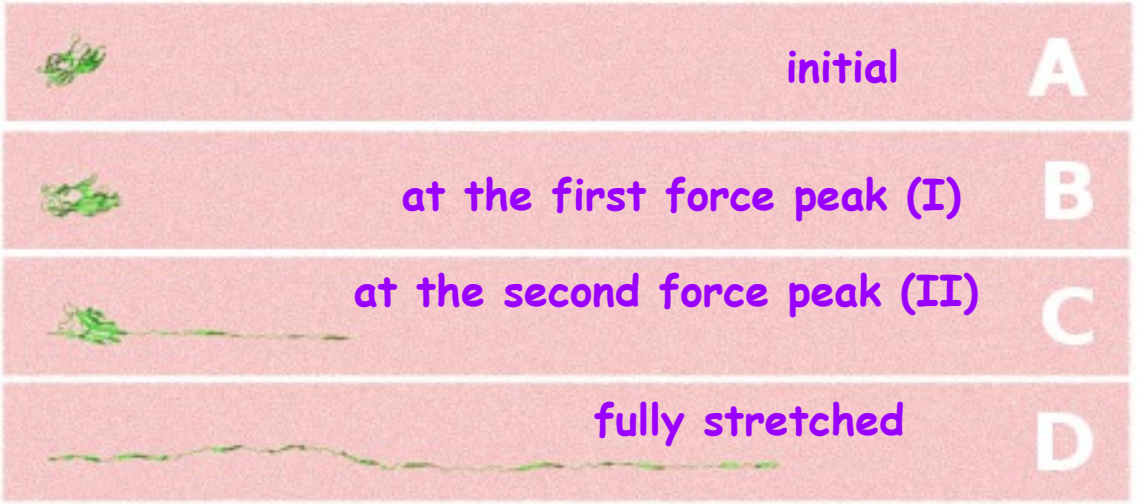
Task 7.3 (example): All-atom SMD of a cohesin domain of scaffoldin from *C. thermocellum*



PDB: 1AOH

Native state of 1AOH after reconstruction of the missing side chains (in red). Reconstruction involved coarse-grained modeling of backbone. There are 4 independent positions of side-chain of 9-Lys amino acid (marked by A, B, C, and D).

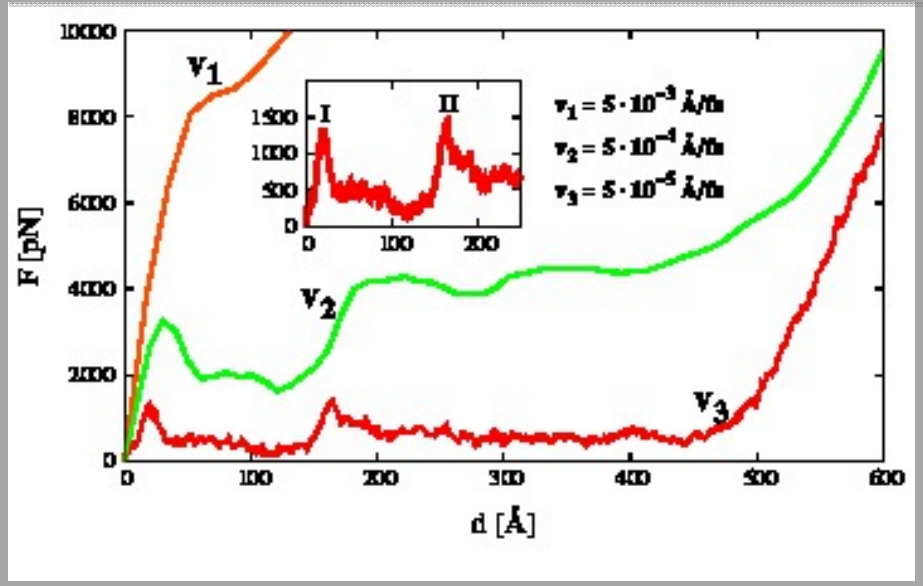
Explicit water, NAMD & CHARMM22



4 stages of pulling with constant speed v

71.5 x 7.5 x 7.5 nm^3

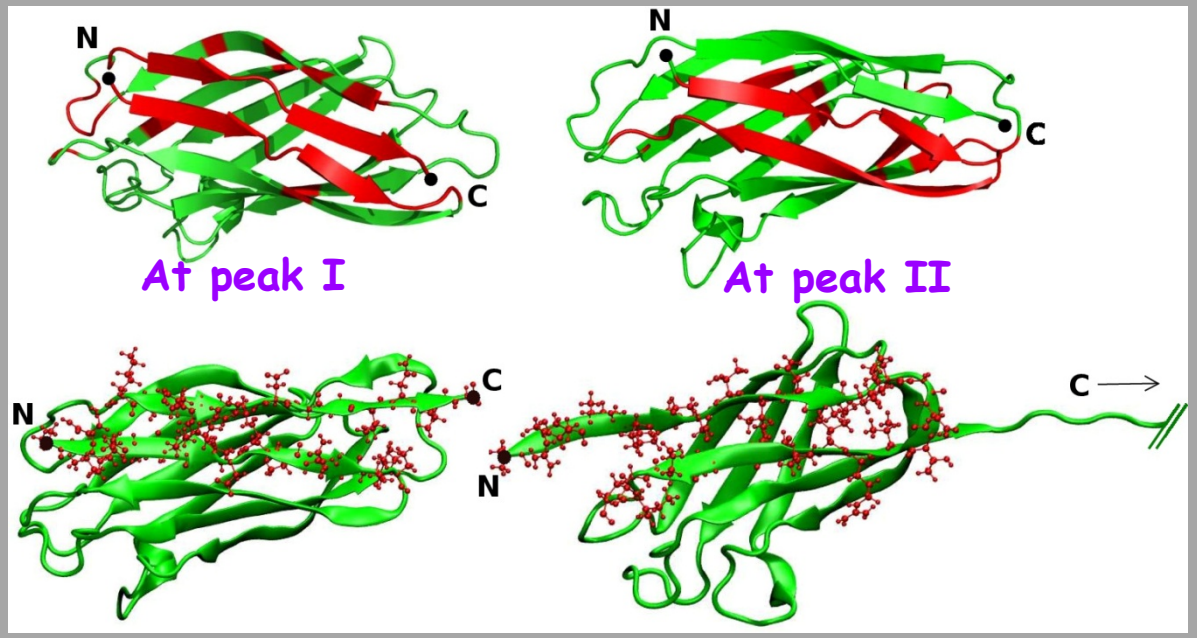
388,455 atoms



Force vs. displacement at the 3 speeds indicated

Several orders of magnitude larger than in experiments – but illustrate the nature of the unravelling

Contacts broken



Large β-sheet network that ruptures in two separate steps

Simulations explain the large forces found in the experiments

General Evaluation

IFPAN with ULIM- Main results:

WP7 Multiscale modeling

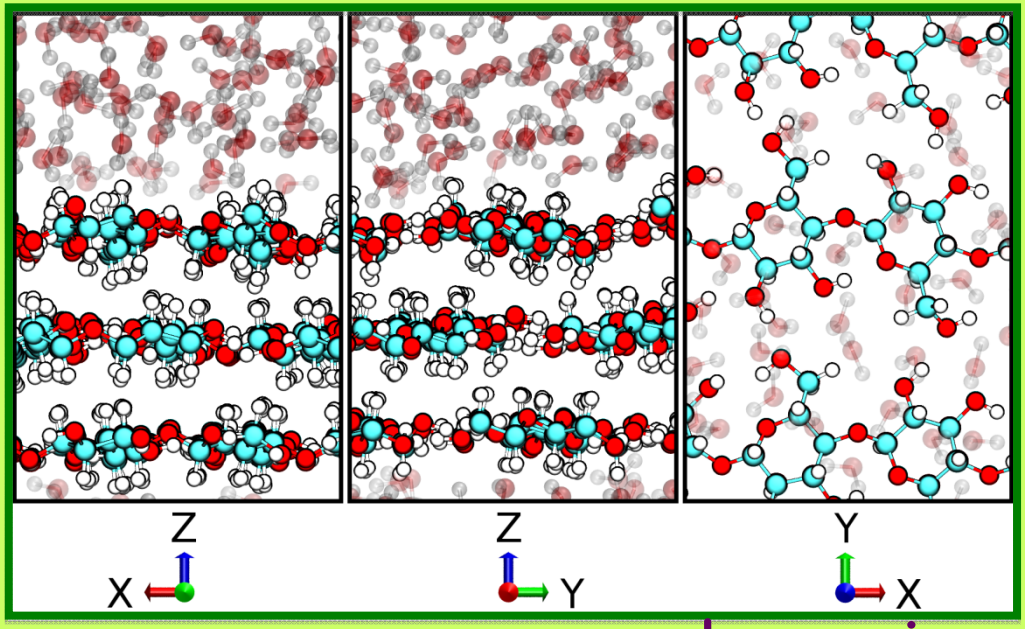
A)Coarse-grained modeling: Providing molecular-level understanding of the mechanical properties of the cohesin-dockerin complexes (shear and tensile mechanical clamps corresponding to various pulling directions). Different cohesins have similar looking force patterns, with different levels of mechanostability, yet when complexed with dockerins they unfold in non-universal ways. Theoretical tests of the mechanical protection strategy in protein nanomechanics.

B)Construction of coarse-grained models of cellulose and of polysaccharide-enzyme complexes. Determination of the model parameters through all-atom simulations, making comparisons to proteins. Providing a theoretical description of the transformation between the crystalline cellulose I-β to cellulose I-α through an amorphous transition state.

C)All-atom simulations: Determination of the peptide recognition capabilities of the crystalline cellulose I-β. Studies of adsorption of dipeptides, tripeptides and small proteins to the cellulose.

D)All-atom-based predictions for single-site mutations that enhance the mechanical and thermal stabilities of cohesin c7A in *C . thermocellum.*

E)Molecular-level interpretation of the SAXS data for the multidomain Xylanase Z in *C. thermocellum*. Characterization of conformational fluctuations in the inter-domain linkers.



Amino acids, dipeptides and proteins at the (100) surface of cellulose Iβ

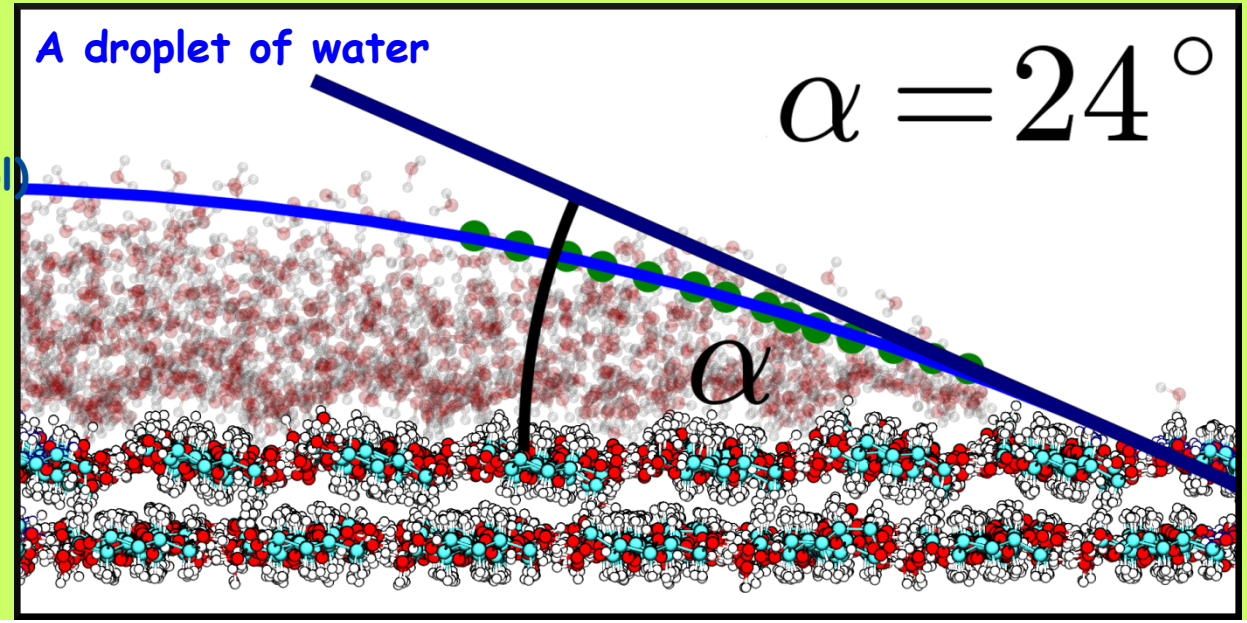
CHARMM36

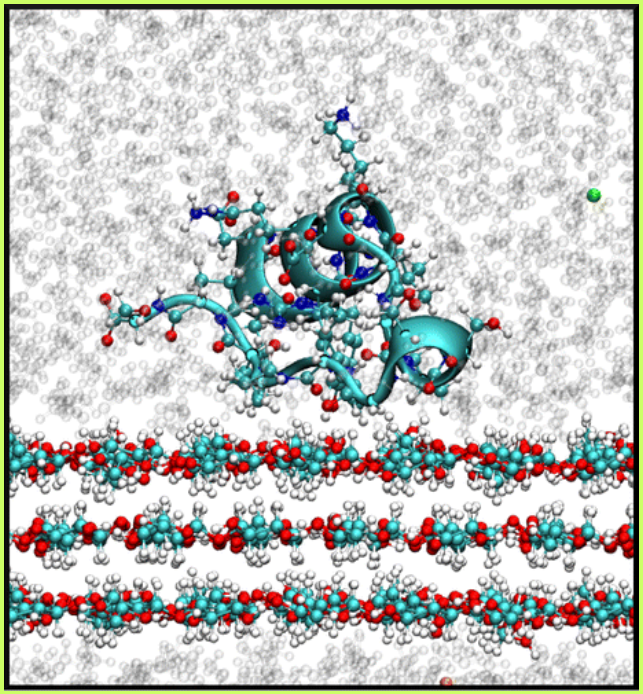
glucose rings raised exposing hydrophilic patches: mildly hydrophilic

Determination of the binding energies of single amino acids

Largest for TYR (10 kJ/mol) then PHE, TRP, MET: aromatic rings

30% specificity



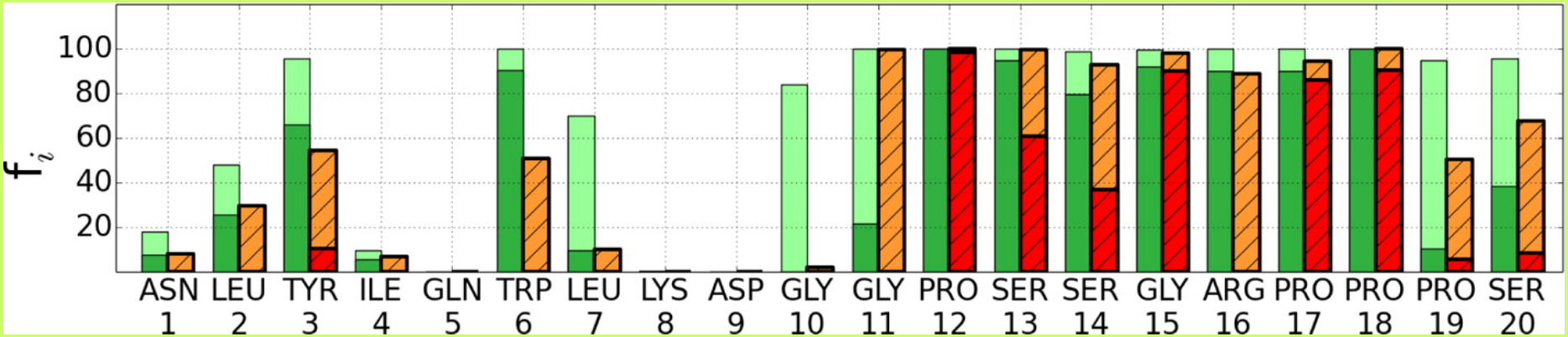


Advanced sampling techniques give full-protein binding profiles

Amino acids, dipeptides and proteins at the (100) surface of cellulose Iβ

CHARMM36

Computed amino acid residence times at the surface: targets to create more cellulose-active mutants of CBM of cellulosome



Peptide Recognition Capabilities of Cellulose in Molecular Dynamics Simulations. Grzegorz Nawrocki, Pierre-André Cazade, Damien Thompson, and Marek Cieplak. J. Phys. Chem. C, 2015, 119 (43), pp 24404-24416

Partner 5 - ABNT

WP 9: Laboratory-scale level integration

Tasks

Task 2.6. Production of substrates. The selected substrates (wheat straw and corn stover) will be produced and supplied to the consortium by ABNT (crude biomass). These could be first subjected to a commonly employed thermochemical pretreatment step by ABNT (pre-treated biomass).

Task 9.3. Pre-industrial scaling up. ABNT will scale the process to pre-industrial level (100 ml flask and 1-5 l reactor) and will perform cost-benefit, life cycle and market analysis of the product and the process developed.

Deliverables

- D7 Report on substrates supply.
- D8 System specifications for pre industrial level.
- D9 Market analysis of the product and the process defined.
- D10 Life cycle analysis.

Objective 1

WP2, Task 2.5

Milestone M3

Deliverable D5

Due to demo plant availability the **substrates (pretreated)** have been only produced recently. In the following weeks they will be distributed to partners, who otherways were not ready for them yet because they were also engaged in other tasks, dealing with non-natural sustrates so far.

This **later suply of the substrate** will assure **short seating of the sample** (short period), **more comparative results** among partners (a single batch: more homogeneus) and **working on the last industrial formulation of the pretreated substrate**.

Contact details

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***Thanks for your attention* !**