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FuturEnzyme:

Technologies of the Future for Low-Cost Enzymes for Environment-Friendly Products

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BEST 18 PRE-LEAD ENZYME MATERIALS OBTAINED AT GRAM SCALE FOR REAL- LIFE TESTS

DELIVERABLE NUMBER D6.1

FABRIZIO BELTRAMETTI
Bio_Ch

Document information sheet

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Authors:	Fabrizio Beltrametti (Bio_Ch), Jan Modregger (Biosynth), Markus Müller (CLIB), Manuel Ferrer (CSIC)
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Contact details:	Fabrizio Beltrametti (fbeltrametti@bioc-chemsolutions.com), Manuel Ferrer (mferrer@icp.csic.es), Jan Modregger (Jan.Modregger@biosynth.com)

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1. Scope of Deliverable

This deliverable consists of a report detailing at least 18 of the best enzymes (recombinant, native, or biomimetic) produced at a multi-gram scale, which partners considered to be sufficient quantities for performing validation tests prior to pre-industrial trials. The material produced was exchanged with or transferred to partners for pre-industrial validation tests.

2. Introduction

This deliverable is a continuation of Deliverable D5.1, 'The Shortlist of at Least 18 Enzymes Nominated for Engineering,' in which a set of enzymes were selected based on laboratory experimental results for their potential scalability and testing in three relevant applications to the project: detergents, textiles, and cosmetics. In this phase, two production methods were chosen: one using *Escherichia coli* and the other using *Pichia pastoris*. For the production phase a total of 41 enzymes have been selected for scale-up production until now (M32), and the details of their production are outlined in Deliverable D6.2. Following the production phase, we proceeded with experimental validation to assess the levels of production and to evaluate the activity and/or stability of the enzymes. This step was crucial before moving forward to their use by our industrial partners.

3. Production of enzymes in multi-gram scale

In WP6, the consortium focused on the production of the different lead candidate enzymes selected in the earlier work packages for application tests. The main target was to supply the best (pre-)lead candidates in gram scale for real-life tests of the industrial consortium partners with application in laundry detergents, cosmetics, and textile.

The initial selection of lead candidates was expanded during the progression of the work packages, since some of the lead candidates turned out difficult to produce in the industrial production host *Pichia pastoris*, which was selected for its capability to secrete the target enzymes into the supernatant. Most of the candidate enzymes were screened in the expression host *E. coli*, which would make industrial application difficult because it expresses the enzymes intracellular. This would require elaborate DSP processes which would be economically not feasible for the intended use in laundry detergents, cosmetics, and textile applications.

In total, 15 lipases/esterases/PETases were selected for further expression and analysis by the industrial partners, and only two of them were scheduled for gram-scale expression but could not be produced in the target scale, either because they did not express well in *Pichia pastoris* or because they did not exhibit any activity after production. All other lipases/esterases were produced in sufficient multi-gram amounts and their activity was confirmed with reference substrates before supplying them to the project partners for further analysis and application tests.

Out of 13 selected hyaluronidases which expressed in *E. coli* or in their native host, only one enzyme could successfully be expressed in *Pichia pastoris*, and the activity of this candidate was confirmed to be rather low. Therefore, the consortium decided that Biosynth will express some reference hyaluronidase enzymes, that are known to be expressed recombinantly. Starting with enzymes which can be produced well and then can be engineered to fulfil the requirements of the industrial partner will be easier than starting with enzymes that fulfil the requirements already from the beginning on but cannot be produced at industrial scale.

In addition to the above enzymes, the consortium has initially selected some peroxidases, laccases and engineered PluriZyme candidates for further analysis, but later removed some of them from the list of high priority candidates.

The strains and methods successfully established optimized in this work package 6 for the production of the enzyme candidates are part of deliverable D6.2 and are summarized the respective document.

4. Verification of Results

For the verification of the results, the successfully produced candidates were supplied to all project partners involved in the isolation and characterization of the respective enzyme family.

The further characterization and confirmation of the lead candidate enzymes is reported in the respective work package, and in the deliverable D6.2.

5. Conclusion

The consortium partners in WP6 have successfully produced the enzyme candidates in the required gram-scale amounts and supplied them to the consortium partners in the other work packages.

While the scale-up and production of the lipases/esterases/PETases has been successful using *Pichia pastoris* for most of those candidates, the remaining enzyme families consisting of hyaluronidases, laccases and peroxidases have been more challenging. Even in these enzyme families, some lead candidates have been produced successfully in gram scale, but some of the more difficult to express candidates are currently still in development.

Since the enzymes have been delivered to the industrial partners in sufficient gram scale amounts, the application tests in work package 7 are currently performed.

The results of deliverable D6.1 are summarized in the table below.

In light of the comprehensive information presented in this deliverable, it is clear that the objectives of the Deliverable D6.1 (or D32) outlined at the onset of this project have been successfully met. A copy of the submitted Deliverable D6.1 has been also recorded in the intranet's project website. See www.futureenzyme.eu -> login -> private-area -> DELIVERABLES & MILESTONES -> DELIVERABLES -> D6.1_Best 18 Pre-Lead Enzyme Materials obtained at gram scale for real-life tests.

BEST 18 PRE-LEAD ENZYME MATERIALS OBTAINED AT GRAM SCALE FOR REAL-LIFE TESTS												
O = oil removal D = dye removal												
ID	Enzyme	Activity	Partner (Origin)	Host (Origin)	Partner (Production)	Host (Production)	Detergent	Textile	Cosmetics	Status	Quantity of produced enzyme	Comment
1	Kest3	Esterase / Lipase	BANGOR		Biosynth	<i>P. pastoris</i>	Yes	No	No	01 - Planned for production		
2	FE_Lip9	Esterase / Lipase / PETase	CSIC	<i>E. coli</i> BL21	Biosynth	<i>P. pastoris</i> and <i>E. coli</i>	Yes	Yes (O)	No	06 - Tested by industry partners	CSIC: 1.05 g BIOSYNTH: 55,7 g (Pichia)	
3	FE_ID 9	Esterase / Lipase	CSIC	<i>E. coli</i> BL21	Biosynth	<i>P. pastoris</i> and <i>E. coli</i>	Yes	No	No	03 - Produced	CSIC: 2.0 g	
4	FE_polur1	Esterase / Lipase / PETase	CSIC		Biosynth	<i>P. pastoris</i>	Yes	Yes (O)	No	04 - Shipped to partners	28,36 g (Pichia)	
5	EstLip_Dim_#008	Esterase / Lipase	UDUS	<i>E. coli</i>	Biosynth	<i>P. pastoris</i>	Yes	Yes (O)	No	06 - Tested by industry partners	41,2 g (Pichia)	
6	EstLip_Paes_TB035	Esterase / Lipase	UDUS	<i>E. coli</i>	Biosynth	<i>P. pastoris</i>	Yes	Yes (O)	No	06 - Tested by industry partners	176 g (Pichia)	
7	EstLip_PtEst1	Esterase / Lipase	UDUS	<i>E. coli</i>	Biosynth	<i>P. pastoris</i>	Yes	Yes (O)	No	04 - Shipped to partners	39,88 g (Pichia)	
8	EstLip_TBec304	Esterase / Lipase	UDUS	<i>E. coli</i>	Biosynth	<i>P. pastoris</i>	Yes	Yes (O)	No	04 - Shipped to partners	93,0 g (Pichia)	
9	Paes_PE-H_Y250S	PETase	UDUS	<i>E. coli</i>	Biosynth	<i>P. pastoris</i>	Yes	Yes (O)	No	06 - Tested by industry partners	94,95 g (Pichia)	additional application in textile's end-of-life
10	PEH_Pbau_PE-H	PETase	UDUS		Biosynth		No	Yes (O)	No	04 - Shipped to partners	125 g (Pichia)	
11	Pform_PE-H	PETase	UDUS	<i>E. coli</i>	Biosynth	<i>P. pastoris</i>	Yes	Yes (O)	No	04 - Shipped to partners	132 g (Pichia)	

12	PEH_Poce_PE-H	PETase	UDUS		Biosynth	P. pastoris	No	Yes (O)	No	04 - Shipped to partners	76,8 g (Pichia)	
13	GEN0105	Esterase / Lipase	BANGOR	Metagenome	Biosynth	P. pastoris	Yes	No	No	04 - Shipped to partners	32, 7 g (Pichia)	
14	GEN0095	Cellulase	BANGOR		Biosynth	P. pastoris	No	Yes	No	03 - Produced	33,4 g (Pichia)	of interest for D if activity confirmed
15	VD-V4_PL9	Hyaluronidase	CNR	Vibrio diabolus	Biosynth	P. pastoris	No	No	Yes	01 - Planned for production		in Pichia; no activity verified
16	VD_PL22	Hyaluronidase	CNR	Vibrio diabolus	Biosynth		No	No	Yes	02 - In production		cloned in Pichia
17	VA_PL9	Hyaluronidase	CNR	Vibrio alginolyticus	BIO_CH		No	No	Yes	02 - In production		cloned in Pichia
18	Hyal_HRDSV_2334	Hyaluronidase	CNR	Halorhabdus sp. SVX81	Biosynth	P. pastoris	No	No	Yes	04 - Shipped to partners	79,4 g (Pichia)	cloned in Pichia
19	VD-V4_GAGL	Hyaluronidase	CNR	Vibrio diabolus	Biosynth		No	No	Yes	02 - In production		cloned in Pichia
20	VA_PL8B	Hyaluronidase	CNR	Vibrio alginolyticus IAMC-CNR#23	BIO_CH	Vibrio alginolyticus IAMC-CNR#23	No	No	Yes	04 - Shipped to partners		low enzyme content in supernatant
21	FE_EH37	Esterase / Lipase	CSIC		Biosynth	P. pastoris	Yes	Yes (O)	No	03 - Produced	51,2 g (Pichia)	
22	FE_Lip5	Esterase / Lipase	CSIC		Biosynth		Yes	No	No	01 - Planned for production		
23	TR2E2	PluriZyme (Esterase / Transaminase)	CSIC		CSIC		Yes	No	No	01 - Planned for production		
24	EH _{1AB1C}	PluriZyme	CSIC		CSIC		unclear	unclear	unclear	01 - Planned for production		
25	X11_mut1	PluriZyme (Esterase / Xylanase)	CSIC		CSIC		Yes	No	No	01 - Planned for production		
26	I8AMQ8	Peroxidase	BANGOR	-	Biosynth	-	No	Yes (D)	No	00 - not investigated further	-	bad evaluation by Bangor
27	Sav1970	Laccase	BANGOR	-	Biosynth	-	No	Yes (D)	No	00 - not investigated further	-	bad evaluation by Bangor
28	[name]	Laccase	BIO_CH		BIO_CH		No	Yes (D)	No	03 - Produced		
29	pVec11	Peroxidase	BANGOR	Metagenome	Biosynth	P. pastoris	No	Yes (D)	No	02 - In production		
30	AB-hyl4	Hyaluronidase	CNR	Planctomycetes sp.	BIO_CH		No	No	Yes	01 - Planned for production		Best Hyaluronidase candidate so far

31	DS_HhyIPL8	Hyaluronidase	CNR	<i>Natronarchaeum sp.</i>	BIO_CH		No	No	Yes	01 - Planned for production		top 5 candidate
32	LC1Hm_4133	Hyaluronidase	CNR	<i>Halomicrobium sp.</i> LC1Hm	CSIC	<i>P. pastoris and E. coli</i>	No	No	Yes	01 - Planned for production		cloned in E.coli by CSIC; top 5 candidate
33	LC1Hm_4133_cut	Hyaluronidase	CNR	<i>Halomicrobium sp.</i> LC1Hm	CSIC	<i>P. pastoris and E. coli</i>	No	No	Yes	01 - Planned for production		cloned in E.coli by CSIC
34	Plct-VS9_Hyl	Hyaluronidase	CNR	<i>Planctomycetes sp.</i>	BIO_CH		No	No	Yes	01 - Planned for production		top 5 candidate
35	BCL_HA12_PG2207 (3)	Hyaluronidase	CNR	<i>Haloferax prahovense</i> BCL_HA12	BIO_CH		No	No	Yes	01 - Planned for production		cloned in E.coli by CSIC
36	BCL_HA12_PG2207 (7)	Hyaluronidase	CNR	<i>Haloferax prahovense</i> BCL_HA12	BIO_CH		No	No	Yes	01 - Planned for production		cloned in E.coli by CSIC
37	LHYAL_HIRNI (Leech hyaluronidase)	Hyaluronidase	Biosynth	Hirudo (leech)	Biosynth	<i>P. pastoris</i>	No	No	Yes	02 - In production		benchmark enzyme
38	HYALP_HUMAN (HP-20 human sperm)	Hyaluronidase	Biosynth	Homo sapiens	Biosynth	<i>P. pastoris</i>	No	No	Yes	3 - In production		benchmark enzyme
39	APIME Hyaluronidase (bee venom)	Hyaluronidase	Biosynth	Apis mellifica	Biosynth	<i>P. pastoris</i>	No	No	Yes	4 - In production		benchmark enzyme
40	HYAL1 (hyaluronidase home sapiens)	Hyaluronidase	Biosynth	Homo sapiens	Biosynth	<i>P. pastoris</i>	No	No	Yes	5 - In production		benchmark enzyme
41	THET1 (Thermobaculum terrenumHyaluronidase)	Hyaluronidase	Biosynth	Thermobaculum terrenum	Biosynth	<i>P. pastoris</i>	No	No	Yes	6 - In production		benchmark enzyme