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FIRST TESTS FOR HYDROLYSIS OF HYALURONIC ACID (HAh)

MS24

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Document information sheet

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FIRST TESTS FOR HYDROLYSIS OF HYALURONIC ACID (HAh)

1. Means of verification

Report available - this milestone will attest the completion and outcomes of the first enzymatic tests for hydrolysing hyaluronic acid.

2. Report available

On 12 September 2023, the Industry meeting Cosmetics #4 online meeting (MS Teams) was organized to discuss about the first round of cosmetic-related tests, particularly the hydrolysis of hyaluronic acid, with pre-selected enzymes. Below, the minutes of this meeting are provided that demonstrated that the milestone was achieved.

Participants:

✓	Fabrizio Beltrametti	BioC-CheM Solutions
✓	Luca Mellere	BioC-CheM Solutions
✓	Jan Modregger	Biosynth (formerly Eucodis)
✓	Markus Müller (protocol)	CLIB
✓	Michail Yakimov	CNR
✓	Patricia Molina	CSIC
✓	Manuel Ferrer	CSIC
✓	Laura Fernandez-Lopez	CSIC
✓	Paula Vidal	CSIC
✓	Xin Lu	Evonik
✓	Carolina Giunta	INOFEA
✓	Carla de Carvalho	IST-ID
✓	Pedro Fernandes	IST-ID

General comments

- Analysis of enzyme activity is difficult for hyaluronidases due to several reasons:
    - Variable substrate: batch-to-batch variability (pH, size distribution) of high and low MW HA (provided by Evonik)
    - Unknown reaction maxima: Newly isolated strains with hyaluronidase activity have unknown optima for growth and/or enzyme reaction. Therefore, it is difficult to target a window of reactive conditions for the enzymes (being different for every isolate/enzyme)
    - Analysis of hydrolysed HA is not yet fully established: Many different analytical methods for measuring the amount and composition of shorter HA fragments were tested. For a detailed analysis, quantitative measurement needs to be established. If proven, qualitative measurements might assist during enzyme production / for activity verification.
- Scaling-up the hydrolysis reaction is needed up to 5-10 g scale of product. This amount of hydrolysed HA is required by Evonik to perform first toxicity and biocompatibility (and formulation) tests.
- If new substrate Hyacare50 or 150 kDa (high MW) is required, please contact Evonik/Xin and Moniec
    - 5-10 g works fast larger quantities might take some weeks to be provided
- Keep list of transferred material up-to-date (OneDrive folder)
    - Also included now: Short list of enzymes with detailed information on optimal reaction conditions and current production status
- List of enzyme candidates is accessible now in OneDrive (\Exchange Project Consortium\ Enzyme Short list and transfers FE partners.pptx)
- Evonik cannot join Zoom meetings due to firewall blockage; as usual, Industry Meetings will continue in MS Teams
- New candidates added to the list (from CNR):

- #30 *Paracoccus sp.* AB-hyl4
- #31 *Natronachaeum sp.* Hyl

Decisions and To Do's

- CLIB finalises Declaration of Results and sends it to partners for feedback
- CSIC and CNR: Enter new strain candidates in short list (Paracoccus and others)
- CNR: Send strains #15-19 (short list) to Bio_CH
- CNR: Optimise cultivation conditions for new strains (#30, #31) to achieve high enzyme activity; CSIC can assist in the analysis if required
- CSIC: Finalise collection of assays for hyaluronidases activity measurement & HA quantitative analysis of degradation products
    - Should be finished as fast as possible because these assays are critical for partners to confirm enzyme activity
- IST-ID: Send hydrolysis samples from two strains to CSIC for HPLC analysis
- All: Please check your internal nomenclature for enzyme candidates and adapt them to the list stated below or in OneDrive. We should all use identical IDs!
- Milestones and Deliverables:
    - MS25: First tests for producing HAh at gram scale (Bio_CH; 09/23)
        - CSIC will take care of it (including the first results they have obtained)
    - MS26: First trials for incorporating HAh into cosmetics (Evonik, 11/23)
        - Not realistic to achieve on time. Postpone to later month; Manolo will contact Colombe to ask for later submission.
    - D7.1 – Report on small/medium validation trials of 18 best pre-selected enzymes (CSIC, 03/24)
        - CSIC in charge of first draft, delivery to partners

Short reports on Objectives

Objective 1: Enzyme production and verification (Biosynth)

- Which enzymes have already been produced? Which ones are next?
    - #15 VD_PL9
- Biosynth has a commercial reference enzyme at hand
- No enzyme activity was yet confirmed in the Pichia produced samples
    - Possibly the salt concentration is too low
- Next enzyme batches will include tags to detect and purify
- CSIC/Manolo will provide conditions of assay that will complement the ones by other partners.
- Candidates #15-18 were already cloned in *Pichia*; low expression level; no confirmed activity
- *Bacillus* and *Coryne* could be tested as alternative hosts

Objective 1: Enzyme production and verification (Bio_CH)

- So far, one strain has been cultivated: #20 *V. alginolyticus* #23
    - 2 x 15 L scale fermentations performed
    - Low protein content (1,5 g/L), low activity
        - Enzyme might be degraded (smear in SDS gel)
        - Glycosylation could also lead to smear; suggestion Jan: use deglycosylation enzymes before putting samples on gel
    - Induction with high-MW HA
    - Suggestion Micha: Test also activity of cells (indicating that enzyme is located intracellular or on membrane)
- New candidates received by CNR? If yes, which will be produced next?
- Liquid and lyophilised supernatant of #20 strain was sent (on the day of the meeting) to partners: Biosynth, IST-ID, CNR, CSIC

- Micha will send remaining strains & protocols to Fabrizio for cultivation/production
    - Short list candidates #15, #16, #17, #18, #19
    - High saline conditions

Objective 1: Enzyme production and verification (IST-ID)

- Paper-based assay is established, verified and transferred to CSIC/Manolo to be implemented in the collection of analytical tools for HA
    - Correlation between MW and viscosity was proven
- Laser-scattering method shows higher variation, less preferred
- Two tested strains originating from IST-ID were tested
    - 703 – *Vibrio sp.*
    - 729 – *Pseudoalteromonas undina*
    - Samples for hyaluronidase activity will be shipped to CSIC for analysis (liquid / on dry ice)
    - When reaction was positive, they will be included in the short list
- Purification of hydrolysed HA after enzyme reaction (question to Xin):
    - Size of enzyme: 30 kDa / size of relevant HA fragments: < 10 kDa
    - Ultrafiltration to remove enzyme; use diluted solution to reduce viscosity
    - Spray-drying or lyophilisation to stabilise HA product (instable in solution)
    - Idea Jan: Use immobilisation of enzyme to simplify purification; Jan will add tags in next round of production (for purification and immobilisation)
- 6 cell strains for hyaluronidase activity could be shipped to CSIC
    - 48 samples of reactions will be shipped for analysis to CSIC; send it on dry ice

Objective 2: Hydrolysis reaction optimisation (CSIC)

- Which enzymes were produced/shipped already by BIO_CH/Biosynth?
- HPLC-based assay has been verified
    - Some candidates show ideal profile of degradation product
- pH 3-7 and 30-50 °C were tested
    - best conditions: pH 5.5, 30°C
- Salt concentration was not varied; should be investigated next
- Anaysis of cells (containing intracellular or membrane bound proteins) and supernatant of new isolates from CNR
    - #30 *Paracoccus sp.* AB-hyl4
    - #31 *Natronachaeum sp.* Hyl
    - Both show good degradation activity and size distribution
        - Will be added to the short list
        - CNR/Micha will optimise the cultivation conditions to achieve higher activities in the supernatant
        - When cultivation protocol is optimised, method will be transferred to Bio_CH for scale-up of the cultivation and the hydrolysis reaction

Objective 3: Scale-up of hydrolysis reaction (Bio_CH)

- Not yet started

Objective 4: Formulation and Application tests (Evonik)

- What needs to be done to achieve MS26?
    - First trials using any kind of enzyme-produced HA, e.g. toxicity or bioactivity assays
    - MS26 will be postponed by CSIC (see above) as no HA is available yet
- What type of application tests would be the first ones when hyaluronic acid hydrolysate is delivered?
- Route at Evonik for new molecules to be implemented in products:
    - Prescreening (3-10 g HA required, can start with 1-2 g)

        - Toxicity analysis
        - Bioactivity (SimDerma platform) on cell cultures
        - First formulation
    - *In vitro* & *in vivo* claim support
        - *In* / *ex vito* studies (human skin models mimicking penetration)
        - *In vivo* studies
        - Marketing & Claim support
    - Scale-up; Production site
    - Launch
- No expectation that toxicity and formulation prescreening is challenging as product is similar to Hyacare50
- Reference product: Hyacare50 as produced at the moment
- For first tests, Evonik will receive only treated HA; when a stable protocol is available for HA hydrolysis and purification, Evonik might also receive strains/enzymes.
    - Will be decided at a later stage
- Delivery of high MW HA (plan with up to 2 weeks until delivery)
    - In house production of HA at Evonik shows batch-to-batch variability (pH 5-8, size distribution, …)
    - Partners should tell how much substrate they need
    - 1st screening should be performed with the same batch of HA
    - When promising enzymes are identified, they should also be test on different batches of HA to check their sensitivity to real batch-to-batch variability

3. Conclusions

Based on the minutes herein provided, we consider Milestone MS24 achieved.