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FIRST TESTS FOR PRODUCING HAH AT GRAM SCALE

MS25

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First tests for producing HAh at gram scale

1. Means of verification

Report available - this milestone will attest the completion of first tests for producing hyaluronic acid products at gram scale.

- Hyaluronidase samples were delivered to the following partners for testing

Table 1. Partners receiving 20 ml of liquid and 450 mg of lyophilized hyaluronate lyase

Short name	Contact name	Phone	Institute/Company
CNR	Dr. Michail Yakimov	+39 090 601 5437 +39 380 792 3856	ISP-CNR
CSIC	Manuel Ferrer	+34 91 585 48 72	Instituto de Catálisis y Petroleoquímica (Consejo Superior de Investigaciones Científicas)
Biosynth	Jan Modregger	+43 189 00804-20	Biosynth GmbH
IST-ID	Carla de Carvalho	+351 21 841 95 94	Instituto Superior Técnico

- both liquid and lyophilized samples were produced and comparable activity was retained

- 1.4 grams of crude secreted hyaluronidase were produced

2. Report available

Identification of a suitable method for the quantification of hyaluronidase

Hyaluronidase activity was qualitatively measured with a modified turbidimetric assay from Rapport, M.M., et al. (Rapport, M.M., Myer, K. and Linker, A. Analysis of the products formed on hydrolysis of hyaluronic acid by testicular hyaluronidase. J Am Chem Soc 73:2416-2420 (1951)). The assay is based on the turbidity resulting from complexes formed between hyaluronic acid and Bovine Serum Albumin. If hyaluronic acid is degraded by the enzyme, reduction in turbidity (OD_{600}) can be observed and measured at the UV-Vis spectrophotometer. The assay consists in two consecutive incubations. The first one aims to hydrolise the hyaluronic acid solution with the hyaluronidase sample under investigation. The second incubation is the turbidimetric reaction between hyaluronic acid and BSA.

To prepare the hyaluronic acid solution 12 mg of Hyacare 100 were solubilized in 40 mL of 300 mM phosphate buffer. After the complete dissolution of Hyacare (achieved after heating the solution at 50°C for 5 minutes), the solution was cooled at 37°C and the pH corrected at 7 with HCl 1N.

Enzyme diluent was prepared by solubilizing 0,9 g of NaCl and 20 mg of BSA in 200 mL of 20 mM phosphate buffer. The pH of the solution was corrected at 7 at 37°C with HCl 1N.

The acidic albumin solution was prepared with 150 mg of BSA in 150 mL of 24 mM sodium acetate. 680 µL of acetic acid were added to the solution and the pH corrected at 3.8 with HCl 6N.

The final procedure of the assay was the following:

1. 375 µL of samples were diluted with 125 µL of enzyme diluent and incubated at 37°C for 10 minutes
2. 500 µL of hyaluronic acid solution were added to the reaction mixture, mixed, and incubated at 37°C for 45 minutes
3. 200 µL of reaction mixture were added to 1000 µL of acidic album solution and the obtained mixture incubated at room temperature for 10 minutes
4. Reduction in turbidity was measured at 600 nm.

Identification of a suitable medium for the production of hyaluronidase

The strain *Vibrio alginolyticus* IAMC-CNR#23 was received by IAMC-CNR of Messina. An initial screening of growth media from *BCSMed Dat* was performed with the strain. The bacterium was grown overnight in Marine Broth (MB) agar plates and then used the obtained sufficient biomass to inoculate different media (100 mL in 500 mL flasks). Growth was carried out at 22°C and 200 rpm. From this initial screening fermentation media BCS365 and BCS366 were identified as suitable for the growth of *Vibrio alginolyticus*.

The selected media were used for initial trials of hyaluronidase production. Liquid cultures of *Vibrio* #23 in BCS365, BCS366 and Marine Broth (as control) were prepared as previously described. Fermentations were run up to 72 hours. Bacterial growth (turbidimetry) and hyaluronidase activity were monitored every 24 hours.

Hyaluronidase activity was assayed by measuring the reduction of the turbidity resulting from complexes formed between hyaluronic acid and BSA. The enzymatic activity (secreted activity) was searched on cultures supernatants obtained after centrifugation of cultures samples at 16000g for 10 minutes. Reduction in turbidity was obtained for all three samples with supernatants from BCS366 cultures being the best (*Figure 1*).

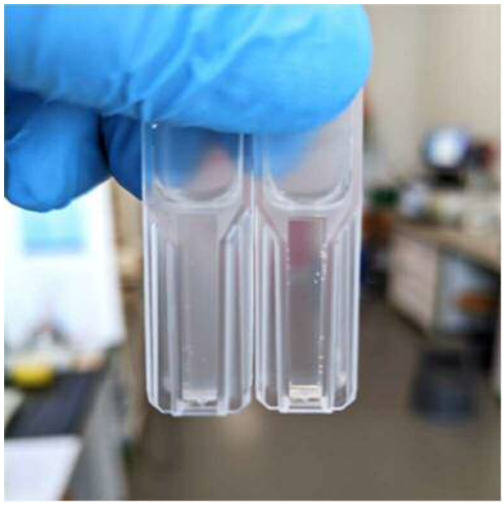

Figure 1: Turbidity assay, blank cuvette with sample treated with water and cuvette with sample treated with BCS366 culture supernatant.

BCS366 grown cultures also gave the best results regarding growth with an OD_{600} of 8.6. For this reason, BCS366 was selected for the optimization of hyaluronidase production (Figure 2).

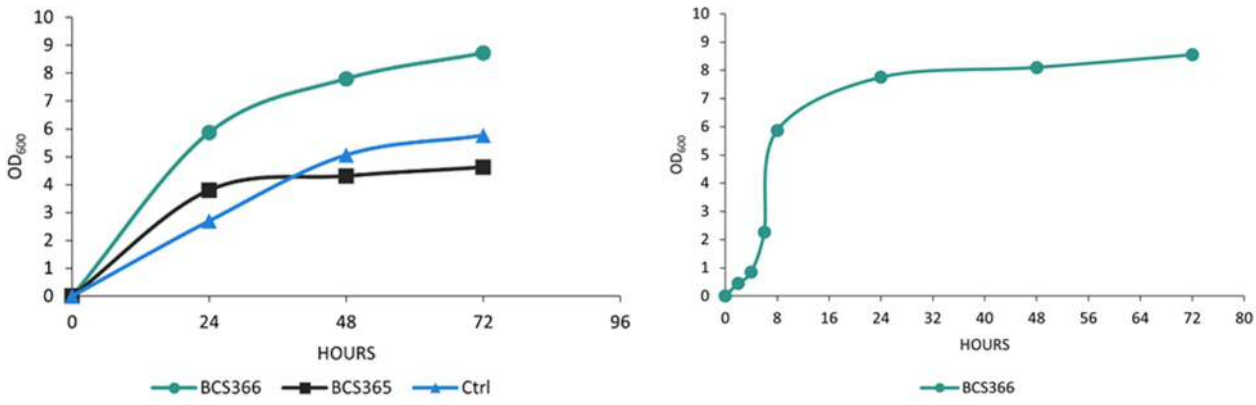

Figure 2: Vibrio #23 *OD_{600} in BCS336, BCS365 and MM (left), and detail of the growth kinetic in BCS366 (right). Data reported are the average of 2 independent experiment.*

Growth at the 15 litres scale of *Vibrio alginolyticus* IAMC-CNR#23 and production of hyaluronidase

A first 15L fermentation was carried out using BCS366 as the production medium. The medium was prepared without hyaluronic acid as inducer. To inoculate the bioreactor four shake flask cultures were prepared using cell loops grown overnight at 25°C on MB agar plates. After 24 hours of incubation at 22°C and 200 rpm, three flasks were pooled together and used as the inoculum.

The fermentation was carried at 22°C and 400 rpm. In these conditions the strain reached an OD_{600} of 10.5 after 20 hours of incubation. The obtained fermentation broth was centrifuged for 60 minutes at 7200g. The supernatants were then pooled together, decanted, and finally filtered through filter paper.

Downstream processing of the secreted hyaluronidase(s)

Ultrafiltration on 30 KDa cellulose membrane was performed to concentrate extracellular proteins. The solution was concentrated 17x. The obtained sample was loaded on SDS-PAGE gels (3.5% acrylamide stacking and 11% resolving) to verify the presence of proteins with molecular weight ranging from 80 to 100 kDa. Coomassie staining revealed the apparent presence of two proteins of 30-37 kDa without any visible bands in the hyaluronidase molecular weight range (*Figure 3*) (despite hyaluronidase activity was observed). It is very likely that hyaluronic acid may be needed in the fermentation broth to better induce the expression of enzymes with hyaluronidase activity. Considering the possibility of having inducible enzymes, shake flask fermentations in the presence of different inducers were performed. In particular, hyacare 100 and 50, cellobiose and chitin were tested for the induction of hyaluronidases in *V. alginolyticus* liquid cultures. Hyacare 100 and 50 were added to the culture medium in the concentration of 0,25 g/L while cellobiose and chitins were tested in the concentrations of 0,25 and 2,5 g/L. Liquid cultures were prepared as described above. The cultures were all incubated at 22°C and 200 rpm besides the cultures with hyacare 100 which were also incubated at 37°C.

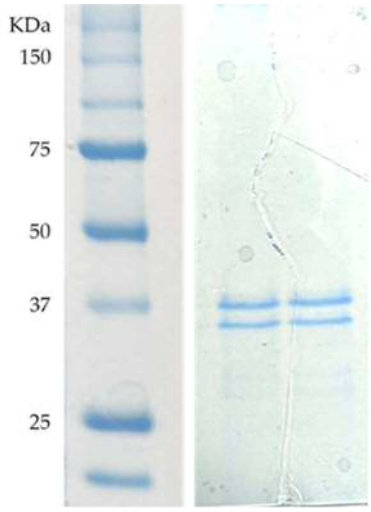

Figure 3: SDS-PAGE analysis of concentrated culture supernatants of Vibrio #23.

Fermentations were carried out for 48 hours. The harvested cultures were centrifugated and supernatants concentrated on 30 kDa Microcon centrifugal filter unit. The 10x concentrated samples were then loaded on SDS-PAGE gels to verify the presence of proteins with molecular weight in the desired range. As evidenced in *Figure 4*, Coomassie staining developed smear for samples 5, 6 and 7 indicating the possibility of having proteins in the desired molecular weight range. Therefore, Hyacare 100 may be the best inducer for hyaluronidase production in *Vibrio #23.* For this reason, pilot-scale production was tested using hyacare 100 as hyalyuronidase inducer.

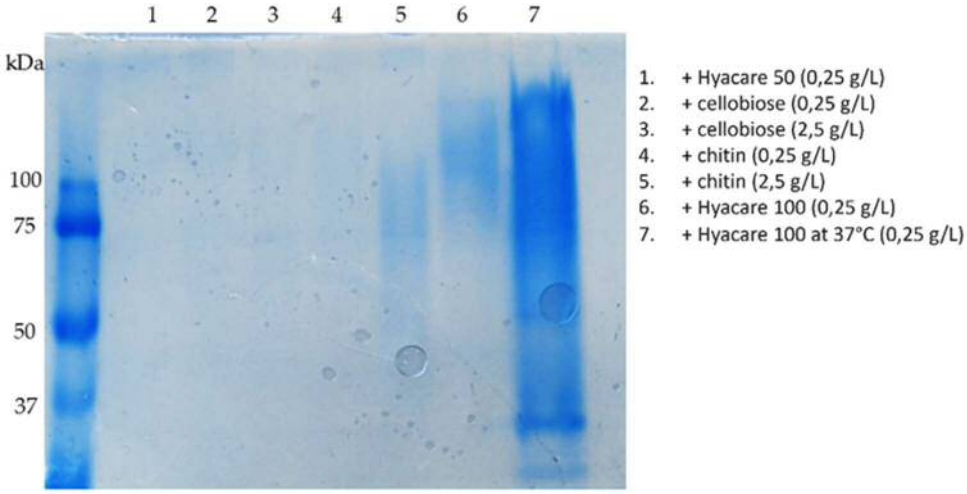

Figure 4: SDS-PAGE analysis of concentrated culture supernatants of Vibrio #23 in the presence of different possible inducers.

Production of hyaluronidase at the 15L scale with hyacare 100 induction

Large scale production was performed using BCS366 and hyacare 100 0,25 g/L as hyaluronidase inducer. For this purpose, 15L of production medium were prepared and sterilized at 121°C for 20 minutes. The inoculum was prepared from 5 100-ml cultures of *Vibrio #23* grown on BCS366 medium for 24 hours at 22°C and 200 rpm. The cultures were pooled together and used to inoculate 15L of BCS366. A solution of 37,5 g/L of hyacare 100 was finally added to the bioreactor to achieve a final concentration of 0,25 g/L. The fermentation was carried out at 37°C and 400 rpm. Bacterial growth was monitored after 20 hours of incubation by means of turbidimetry. In this condition, the OD_{600} reached resulted of 7.5. To avoid possible cell lysis the 15L culture was therefore harvested.

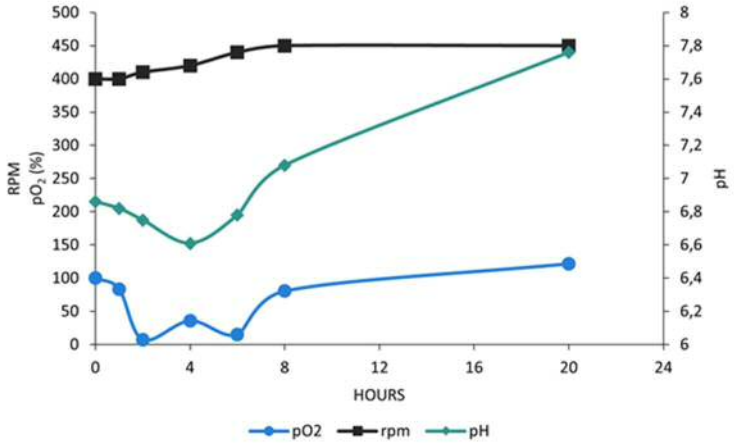

Figure 5: fermentation trends of rpm, pH and pO_2.

Harvest

The separation of the bacterial cells from the supernatant was performed at 7200g for 60 min. The hyaluronate lyase-containing supernatant was then decanted and filtered through filter paper prior to ultrafiltration (see below).

Concentration of the supernatant

Concentration of the supernatant containing the hyaluronate lyase activity was performed through ultrafiltration on 30 kDa cellulose membrane. The concentration achieved was 23x. The resulting concentrate appeared to be very viscous probably due to the presence of residual hyaluronic acid. It was interesting to note that the viscosity did not decrease over time suggesting partial dilution of HA and the presence of stable degradation products.

SDS-PAGE analysis

SDS-PAGE analysis was performed on denaturing gels 3.5% acrylamide stacking and 12% resolving. As shown in *Figure 6*, in the size range expected for hyaluronate lyase, a protein smear appeared. This could be due to the presence of residual hyaluronic acid or to the glycosylation of the hyaluronate lyase(s). High salinity of the sample was excluded as the same result was obtained upon precipitation of the protein with 70% ammonium sulphate. The treatment of the secretome with glycosidases is in progress to elucidate this issue.

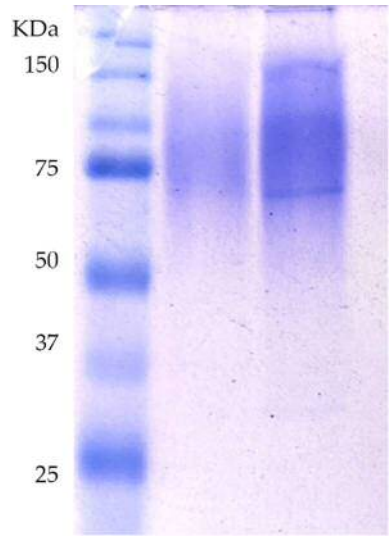

Figure 6: SDS-PAGE analysis of culture supernatant (1), protein concentrate 23x (2).

To reduce its viscosity, the solution was gradually acidified with HCl 6N to hopefully precipitate the residual hyaluronic acid. From pH 4 to pH 1 precipitates were gradually obtained. The obtained samples were then centrifuged, and supernatants loaded on SDS-PAGE gels. Protein smears disappeared from samples collected at acidic pH (4 to 1) while remained present in samples at pH 5, 6, 7 and 8.

Delivery of samples and verification to partners

Samples produced as above were delivered to selected partners according to Table 1. Samples were bot in liquid and Lyophylized form. To date, samples were verified by IAMC-CNR with a qualitative test (Figure 7). It in interesting to note that the activity was retained both in the liquid (4°C storage) and lyophilized samples.

Table 1. Partners receiving 20 ml of liquid and 450 mg of lyophilized hyaluronate lyase

Short name	Contact name	Phone	Institute/Company

CNR	Dr. Michail Yakimov	+39 090 601 5437 +39 380 792 3856	ISP-CNR
CSIC	Manuel Ferrer	+34 91 585 48 72	Instituto de Catálisis y Petroleoquímica (Consejo Superior de Investigaciones Científicas)
Biosynth	Jan Modregger	+43 189 00804-20	Biosynth GmbH
IST-ID	Carla de Carvalho	+351 21 841 95 94	Instituto Superior Técnico

Figure 7: Qualitative test for the activity of hyaluronate lyase performed by IAMC-CNR (L: liquid hyaluronate lyase; B: lyophilized hyaluronate lyase).

Hydrolysis of hyaluronic acid and production of degradation products

CSIC tested the material sent by BIOC-CHEM (both concentrated supernatant sample, and lyophilized material), under the following reaction conditions:
Total volume: 200µl; Time: 72h; Temperature: 37°C
Reaction concentrations:
In BR buffer pH 4.0, 5.0, 6.0, 7.0, 8.0, 150mM NaCl (pH7.0) and 300mM NaCl (pH7.0)
Sb → 20µL in reaction
Lyophilisate → 0.5µg in reaction
HYACARE (HA) → 1g/L in reaction
HYACARE50(HA50) → 2g/L in reaction

Maximal activity was found at pH 7.0 without any other supplement, for example, NaCl (see Figure 7). As shown in Figure 7, the hyaluronidase was particularly efficient for the degradation of and polymeric hyaluronic acid HA when tested at a concentration of 1 g/L.

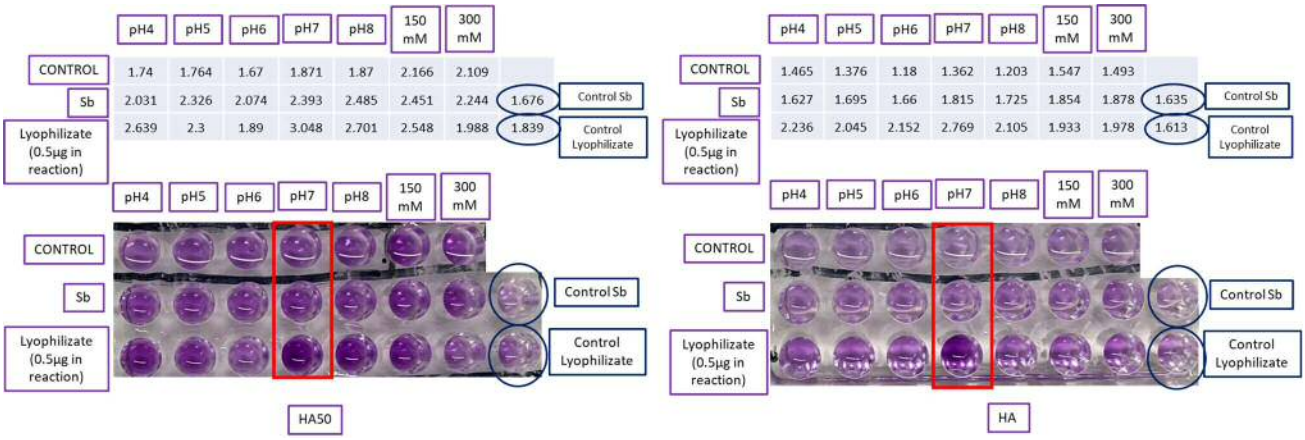

Figure 8: Quantitative test for the hydrolysis of partially hydrolyzed hyaluronic acid HA50 and polymeric hyaluronic acid HA in the presence of hyaluronate lyase provided by IAMC-CNR and BIOC-CHEM *(Sb: liquid hyaluronate lyase; Lyophilizate: lyophilized hyaluronate lyase).*

3. Conclusions and outlook

The production of hyaluronidase from *V. alginolyticus* was performed in medium BCS366. The overall yield in secreted proteins (of which the hyaluronidase represented the most abundant proportion) was of approximately 0.1 g/L for a total productivity of 1.4 grams. The hyaluronidase activity detected allowed the degradation of 1 g/L hyaluronic acid to an extent suitable for the verification of the resulting hyaluronic acid digestion products. Improvement of the productivity and of the assay method are in progress. Based on the results presented in this document, we consider Milestone MS25 achieved.