Use of DENARASE® for tomorrow´s industry

Background & Motivation to use DENARASE®

The removal of nucleic acids from biological samples is a necessary step for applications in Red Biotechnology and Molecular Biology. Along the production of biopharmaceuticals during fermentative processes – for instance, proteins, antibodies, vaccines, etc. –, nucleic acids are generated and accumulated in the culture broth, particularly when microbial cells are disrupted during the work-up, and intracellular material is poured into the aqueous phase. The accumulation of nucleic acids increases the viscosity of the liquid fraction significantly and complicates the downstream processing and the purification steps. Moreover, to be marketable, and based on strict safety regulations, biopharmaceutical products need to be almost free of nucleic acids.

Conclusively, a nucleic acid removal step is mandatory before commercialization. To tackle that need, traditional approaches comprise sonication, precipitation, or extraction. Though effective, these options often need relatively severe processing conditions (e.g. addition of chemicals or solvents, temperature, pressure, etc.), and thus a partial degradation of the targeted biopharmaceuticals may be expected during the nucleic acid removal step. As an alternative, nucleic acids may be removed upon hydrolysis by means of unselective nucleases, which are hydrolytic enzymes that cleave the phosphodiester bonds of nucleic acids yielding smaller oligonucleotides of several base pairs.

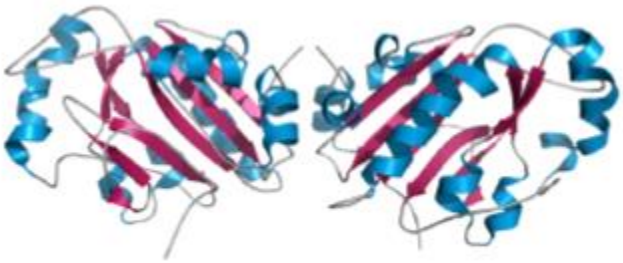
An excellent enzyme candidate is the endonuclease secreted by *Serratia marcescens*. This endonuclease displays high catalytic activity with a remarkable broad substrate range, efficiently cleaving all forms of nucleic acids, RNA and DNA, single- and double-stranded, as

well as linear or circular sequences. The final products are oligonucleotide fragments of 2-5 base pairs. Moreover, the *S. marcescens* endonuclease shows high stability, remaining active upon the addition of deleterious agents like ionic, non-ionic, chaotropic agents, or denaturing compounds like urea.

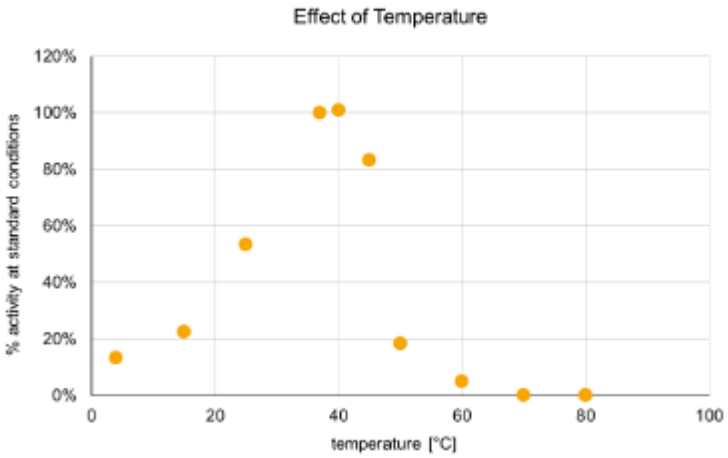
Based on its outstanding performance a genetically-engineered form of *Serratia marcescens* endonuclease – the so-called DENARASE® – has been launched by c-LEcta GmbH (Leipzig, Germany). DENARASE® keeps the expected excellent broad operational window of pH and temperature of the endonuclease of *S. marcescens*, and besides that, it offers other selling points. The patented production microorganism is not *Escherichia coli*, but the Gram-positive *Bacillus* sp., which is known to be an endotoxin-free strain. The employed fermentation media is free of animal-derived feedstocks and antibiotics, and therefore DENARASE® may be considered a BSE/TSE-free product, with a high viral safety (as no animal derived materials are involved in its production). Furthermore, DENARASE® does not display proteolytic activity, and thus does not degrade peptides. This may be relevant when proteins are the marketable targets of the fermentation. Adding to these facts, DENARASE® can be delivered at high quantities and with an excellent quality price ratio. Finally, it must be noted that DENARASE® manufacturing process is in full compliance with the cGMP requirements. Overall, this may open novel markets for endonucleases and may also trigger their use in already established applications, such as medical and (bio)pharmaceutical manufacturing, cell-therapy, oncology, car-t-cell development, etc.

Product & Specifications of DENARASE®

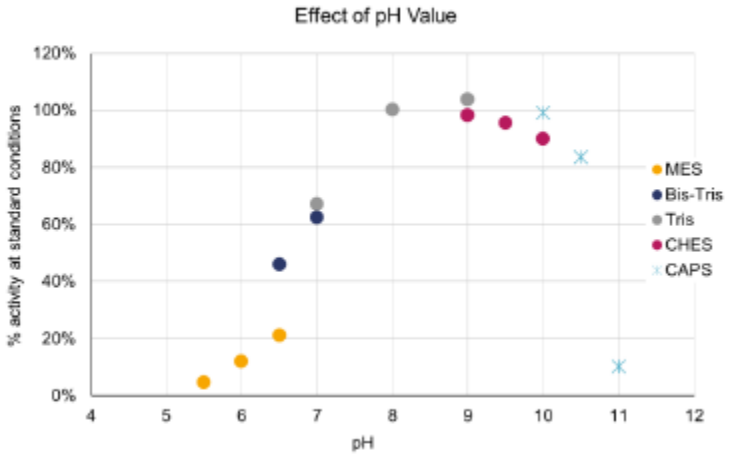
As stated above, DENARASE® is a genetically engineered endonuclease originating from *Serratia marcescens*. The enzyme catalyzes the hydrolysis of phosphodiesters of single and double stranded, linear, and circular forms of DNA and RNA into smaller oligonucleotides of 2-5 base pairs. The enzyme consists of two protein subunits (each 27 kDa).



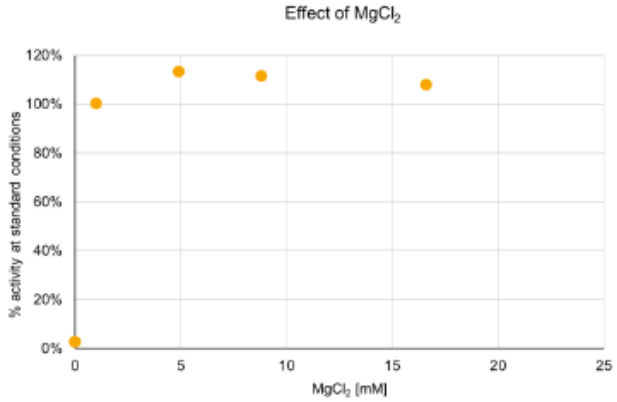
DENARASE® shows an optimum temperature of 37ºC. However, it remains active in a much wider range (0-42 ºC) what enables its use in many practical conditions at different temperatures.

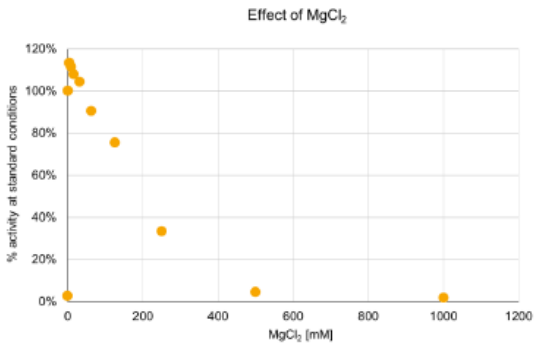


Likewise, DENARASE® shows a broad pH range of activity, reaching its maximum in the range of 8-10, regardless the type of buffer employed (MES, Bis-Tris, Tris, CHES, CAPS). These results are a further demonstration of the robustness of the enzyme, able to adapt to many processing conditions.

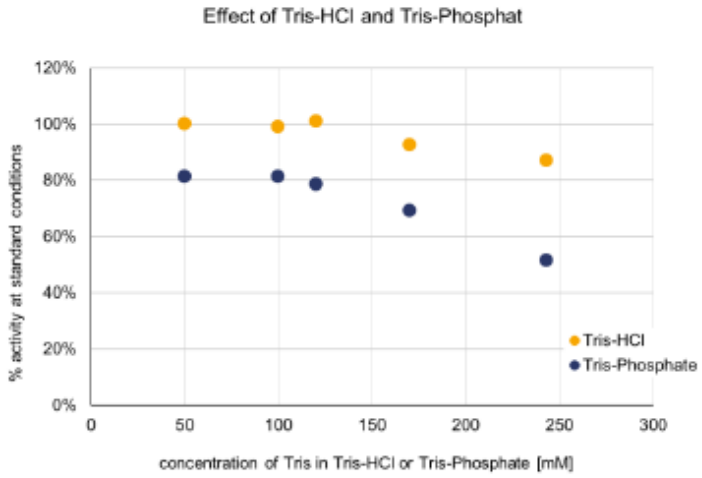


For its performance, DENARASE® requires Mg^{2+} as cofactor (1-2 mM). Moreover, the enzyme remains fully active at much higher concentrations of $MgCl_2$ (up to 100 mM). At higher concentrations (> 150 mM), a deleterious effect is observed.

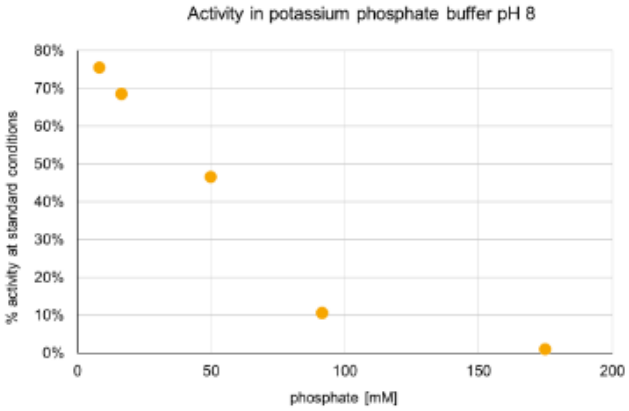


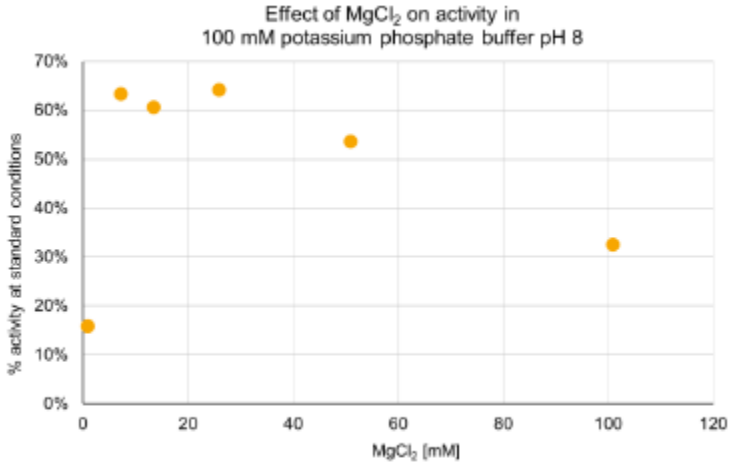


DENARASE® displays activity over different buffers with a broad range of concentrations and ionic strengths. Thus, the use of tris-HCl buffer leads to outstanding results, and DENARASE® displays full activity until buffer concentrations of 150 mM. When the buffer media turns to tris-phosphate, results are positive, yet only 80 % activity is reached at 50 mM.

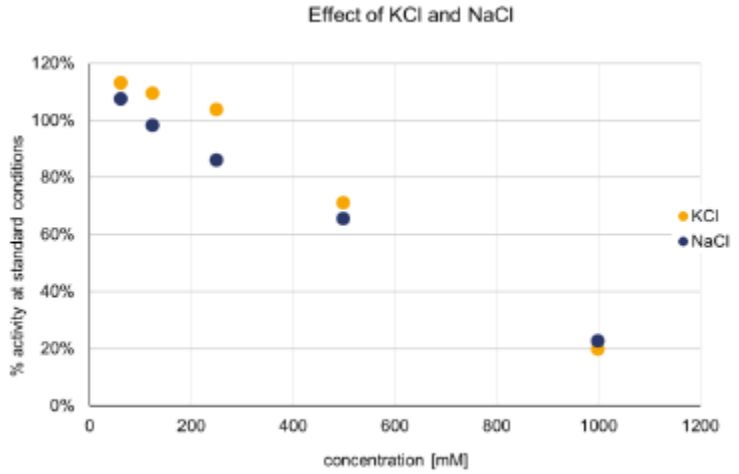


With potassium phosphate (pH 8), the ionic strength must be kept low (range of 5-10 mM) to secure 80 % activity. However, this can be broadened when the buffer is combined with $MgCl_2$. Thus, at concentrations of 20-40 mM of $MgCl_2$, DENARASE® retains 60-70 % of its activity at 100 mM phosphate buffer.

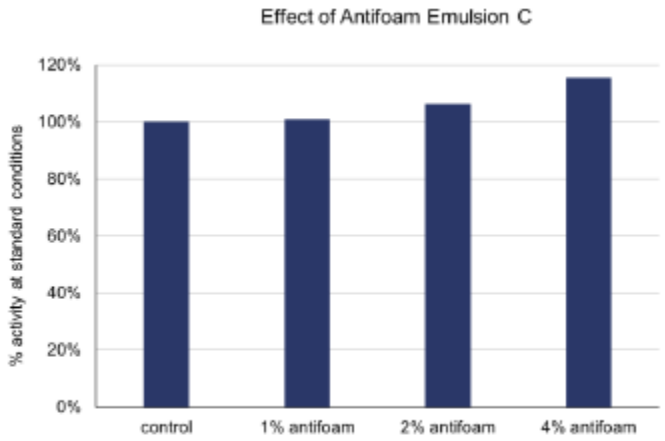




The presence of halide salts (namely KCl and NaCl) may be frequent in fermentative broths. Remarkably, DENARASE® can keep virtually full activity at concentrations of up to 300 mM of these salts. This highlights once more the capability of the nuclease to display nucleic acid removal under diverse conditions.

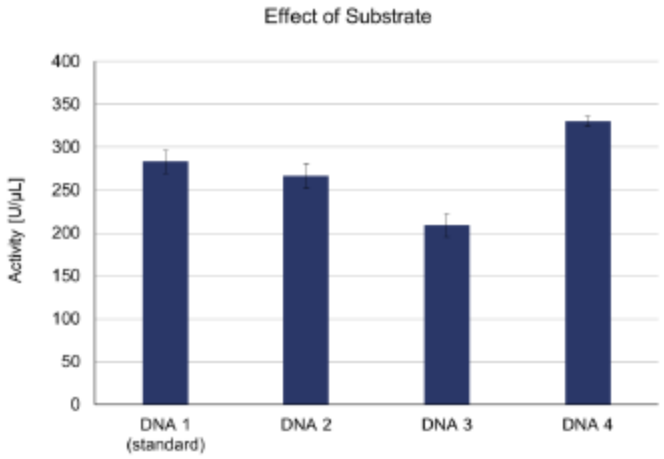


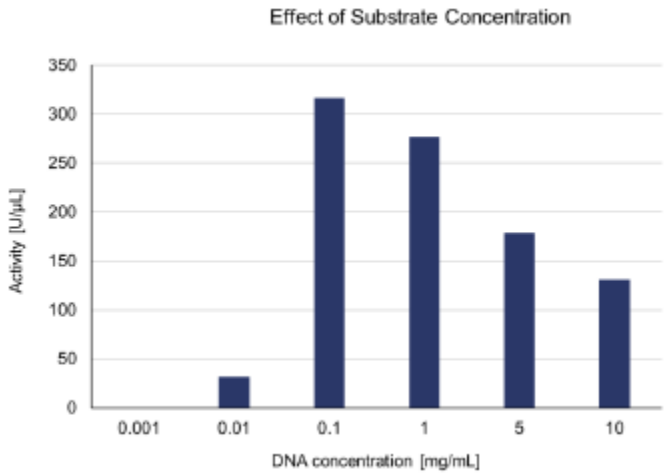
Apart from buffers, salts and ionic strength, real conditions at industries – when nucleases are needed – often include the presence of other compounds that may lead to foam and emulsion formation. To proceed with downstream units properly, the addition of antifoaming agents may be needed in some cases. Notably, DENARASE® can accept these compounds, not only keeping its full activity in nucleic acid removal, but also slightly increasing it at higher antifoam concentrations (4 %).



DENARASE® activity

As stated above, DENARASE® can cleave many form of nucleic acids (linear, circular, double branded, etc.). To characterize the biocatalyst, one unit of DENARASE® is established as the enzyme amount that causes a change of one Absorbance Unit at 280 nm in 30 minutes. It must be noted, however, that vendor specifications for the activities of various commercially available endonucleases cannot be compared due to differences in the activity assays, e.g. substrate concentration and substrate origin that were used.





DENARASE® Specifications

Parameter	Method	Specification
Appearance	Visual	Clear, transparent solution
Activity	Photometric	> 250 U / µL
Purity	Protein purity determined by SDS-PAGE and silver staining	> 99 %
Specific activity	Activity per protein content determined photometrically at 280 nm with a molar extinction coefficient of 44.	> 6 x 10^5 U / mg
Protease activity	Protease detection assay	No protease activity detectable
Endotoxin level	LAL-Test Ph. Eur. 2.6.14 Method C	< 0.25 EU / KU
Total microbial count	TAMC / TYMC Ph. Eur. 2.6.12	Aerobic bacteria: < 5 cfu / 200 µL Yeast / moulds: > 5 cfu / 200 µL

SHIPMENT, TRANSPORTATION AND STORAGE OF DENARASE®

DENARASE® is supplied in a stabilizing glycerol solution, which is composed as follows: 50 % glycerol (v/v) (synthetic origin), 20 mM Tris-HCl, pH 8.2 / 20 mM NaCl / 2 mM $MgCl_2$. The product is filled in non-pyrogenic, USP Class VI, polypropylene vials. During transportation the vials are packed in polystyrene boxes with pre-cooled cooling units.

The DENARASE® order size is:

- 100 KU / 500 KU (produced under cGMP and filled under ISO9001).
- 1 MU / 5 MU (produced and filled under cGMP).

The ideal shipment temperature is at -20ºC. However, DENARASE® results to be a very robust derivative, and thus it can keep full activity under different shipping conditions (see below). Whereas the optimal storage temperature is – 20ºC, it is not recommended to store the product at temperatures below – 20ºC, because the freezing and thawing of the product may lead to a significant loss of activity.

STABILITY ANALYSIS OF DENARASE® - A SHIPPING STRESS TEST

As stated above, to cope with industrial applications, enzymes must be robust and stable. The assessment of stability of DENARASE® at -20 ºC clearly demonstrates its outstanding stability. Two batches of DENARASE® were produced and stored at -20ºC, with regular measurements of the activity. Gratifyingly, no losses of activity were observed even at 25 months of storage.

DENARASE® BATCH	INITIAL ACTIVITY	STORAGE TIME	REMAINING ACTIVITY
Batch 1	308 U μL^{-1}	12 months	308.4 U μL^{-1}
Batch 2	284 U μL^{-1}	25 months	316 U μL^{-1}

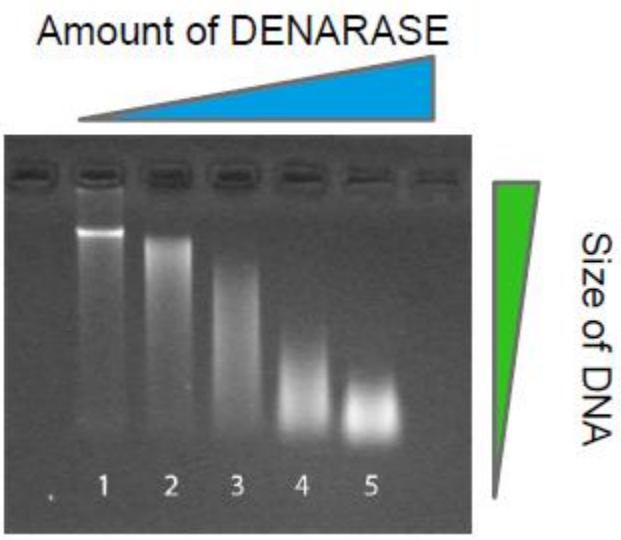
In addition to this study, simulations on shipping conditions were performed. Several volume samples of DENARASE® were stored at different temperatures (from -20ºC to 30 ºC) during some days. Enzymatic activities were then recorded. Results are summarized in Table, and clearly emphasize the robustness of the enzymatic derivative.

INCUBATION CONDITIONS	SAMPLE VOLUME	REMAINING ACTIVITY ($U\ \mu\ L^{-1}$)
Initial Activity DENARASE® batch	---	260.36 ± 7.25
10 days at 30ºC	0.5 mL	264.29 ± 15.28
10 days at 30ºC	2 mL	271.34 ± 8.08
10 days at 30ºC	30 mL	276.58 ± 4.03
1 day at 30ºC, then 1 day at 4ºC, then 1 day at 20ºC, then 1 day at -20ºC	0.5 mL	265.50 ± 8.59
1 day at 30ºC, then 1 day at 4ºC, then 1 day at 20ºC, then 1 day at -20ºC	2 mL	261.59 ± 8.27
1 day at 30ºC, then 1 day at 4ºC, then 1 day at 20ºC, then 1 day at -20ºC	30 mL	267.62 ± 7.27

PRACTICAL CASE STUDIES FOR DENARASE®

The main application for DENARASE® is the DNA/RNA removal of biological and biopharmaceutical samples, prior to their commercialization free of nucleic acids. As stated above, this may be performed due to the broad acceptance of DENARASE® for all kinds of DNA and RNA.

In this respect, for the manufacture of recombinant biologicals for therapeutic use, the FDA sets nucleic acid levels below 100 pg per dose (end product). The contamination level is determined by sensitive DNA assays, which rely on hybridizable nucleic acids. Given the fact that DENARASE® degrades nucleic acids to oligonucleotides (2-5 base pairs), the hybridization is no longer possible, and thus compliance with regulatory requirements may be assured.



DENARASE® can be used when:

- **Reducing Viscosity.** Cells are disrupted during the manufacture of recombinant biologicals, and macromolecular nucleic acids are released to fermentation broth, generating a sharp increase in the viscosity of the effluent. This fact complicates the downstream processing significantly. Herein, the addition of the endonuclease degrades rapidly the nucleic acids, thus diminishing the viscosity tremendously. This is very helpful, especially when filtration or other purification steps come subsequently in the production pipeline. It reduces the processing time, and increases the yield, as endonucleases do not affect the product titers (contrary to other more severe methods for nucleic acid removal).

- **Particle processing.** Nucleic acids may adhere to biologically produced particles, such as viruses or inclusion bodies, what impairs product features and usability. The use of DENARASE® may solve these problems.

- **Bioanalytic applications.** There are a number of bioanalytic methods that require highly pure sampling materials (e.g. ELISA, 2-D electrophoresis, chromatography, etc.). A pretreatment of these samples with DENARASE® may become a useful strategy.

HOW TO START USING DENARASE®?

Once DENARASE® is received at the lab, first trials are important to set the proper catalytic conditions for the enzyme in the biological media of interest. It must be noted that the activity of DENARASE® may be influenced by a variety of parameters (e.g. temperature, pH, buffer composition, ionic strength, etc.), as discussed in this document. Conclusively, the optimal amount of DENARASE® that is needed for a given application, must be determined on a case by case basis.

EXAMPLE. Performing the reduction of viscosity of an *Escherichia coli* process solution. The solution contains 300 g *E. coli* biomass / Liter of 50 mM potassium phosphate buffer pH 7. As starting point, the addition of 20 KUnits of DENARASE® per liter of process solution – after cell disruption – is recommended. Subsequently, an incubation of 30 minutes at room temperature is established. If less DNA and RNA are present, or temperatures close to 37ºC can be chosen (optimal for DENARASE®), then less enzyme loadings can be assessed. Conversely, if process conditions result inhibitory for DENARASE®, higher enzyme loadings, longer incubation times (or both) should be set.

Frequently asked questions (FAQ) about DENARASE®.

- How DENARASE® is removed from the process or the final product respectively?

A variety of methods is available for the removal of DENARASE® during the downstream-processing. However, best method depends on the individual process. In many cases the processes involve chromatography steps in which removal of DENARASE® can be easily implemented (e.g. ion exchange, hydrophobic interaction and size exclusion chromatography). As an alternative, ultrafiltration can be applied for separation of DENARASE® from target molecules. The efficiency of removal can be verified by a commercially available ELISA-kit.

- How to prove absence of DENARASE®?

The efficiency of removal can be verified by a commercially available ELISA-kit.

- How much DENARASE® must be used in the application?

General recommendations can be provided to start with the process. However, since optimum conditions will vary from product to product, for highest efficiency it is strongly recommended to determine those experimentally.

- At which process step should DENARASE® be applied?

This depends on the individual process. In general, one good step is after the fermentation and disruption of the cells, before other filtration and separation steps.

- How can DENARASE® be inactivated?

Since DENARASE® depends on Mg^{2+} it can be inhibited by chelating the divalent cations with EDTA.

- Is it possible to use immobilized DENARASE®?

Unfortunately not. So far, all efforts to bind such endonuclease to a support did not succeed, mainly due to the macromolecular nature of the substrate.