Rapid biotransformation and inactivation of subtilisin and α-amylase in wastewater treatment plants

Author	Affiliation	Email address
Evanthia Bournaka*	DHI, Agern Allé 5, 2970 Hørsholm, Denmark	EVBO@dhigroup.com
Michael Thorsen	Novozymes A/S, Krogshøjvej 36, 2880 Bagsværd, Denmark	MITH@novonesis.com
Bettina Margrethe Jensen	Novozymes A/S, Krogshøjvej 36, 2880 Bagsværd, Denmark	BMJ@novonesis.com
Torben Madsen	DHI, Agern Allé 5, 2970 Hørsholm, Denmark	no current institutional email address

**Corresponding author*

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Evanthia Bournaka*

DHI, Agern Allé 5, 2970 Hoersholm, Denmark

*Corresponding author. E-mail: EVBO@dhigroup.com

https://orcid.org/0000-0002-2556-4465

Michael Thorsen

Novozymes AS, Krogshoejvej 36, 2880 Bagsvaerd, Denmark

E-mail: MITH@novonesis.com

https://orcid.org/0000-0001-8735-3893

Bettina Margrethe Jensen

Novozymes AS, Krogshoejvej 36, 2880 Bagsvaerd, Denmark

E-mail: BMJ@novonesis.com

https://orcid.org/0000-0002-7722-4040

Torben Madsen

DHI, Agern Allé 5, 2970 Hoersholm, Denmark

https://orcid.org/0009-0000-1385-4804

ABSTRACT

This study quantified the removal and biotransformation of the industrial enzymes subtilisin and α-amylase in wastewater treatment plants (WWTPs) and activated sludge systems. Measurements of subtilisin and α-amylase at an industrial WWTP showed high inlet concentrations and concentrations below quantification limits in the outlet, corresponding to removals of more than 99% of intact enzymes. Lower concentrations of the enzymes were found in the inlet to a municipal WWTP at which the concentrations in the outlet were below the quantification limits. Complementary laboratory simulation experiments with activated sludge demonstrated extremely rapid biotransformation of subtilisin and α-amylase under aerobic conditions. More than 70% of the intact enzyme protein was lost within 0.25 min, and more than 90% within 5 min, followed by slower continuous transformation to levels below quantification limits within 48 h. The transformation followed first order kinetics with Disappearance Time 50 (DT50) values of less than 10 seconds for both enzymes. In contrast, only minor losses were observed in abiotic sludge, indicating that microbial degradation is the primary removal mechanism. Overall, the results show that subtilisin and α-amylase are rapidly transformed during wastewater treatment, supporting the assumption of very low environmental concentrations and providing robust data for environmental exposure modelling.

Keywords: activated sludge system, amylase, biotransformation rate constants, environmental exposure prediction, enzymes, subtilisin

HIGHLIGHTS

- Removal of more than 99.9% of subtilisin and more than 99.0% of α-amylase in wastewater treatment plants
- Activated sludge simulation tests support field observations

- Activated sludge tests demonstrate very rapid initial transformation of subtilisin and α-amylase, and time for 50% enzyme transformation (DT50) was less than 10 seconds
- Abiotic controls indicate microbial degradation as the primary removal mechanism

INTRODUCTION

Enzymes are globular proteins with an active site. They act as biological catalysts and are widely used in societies around the globe to optimize a multitude of different processes. Subtilisin (protease) is a proteolytic enzyme that degrades proteins and has broad applications in areas such as laundry and dishwashing detergents, production of food and beverages, animal feed production, and leather processing (HERA 2007; Jegannathan & Nielsen 2013). In contrast, α-amylase is a non-proteolytic enzyme that catalyzes the hydrolysis of starch into simpler sugars and is widely used in laundry and dishwashing detergents and in the production of cereals-based food products, such as beer and bread (HERA 2005; Jegannathan & Nielsen 2013).

The amount of a chemical substance emitted to surface water is determined by its volume of use, the emission pathway and the physicochemical properties. Enzymes represent high volume substances, as the total volume of manufactured and imported final enzyme products is approx. 230,000 tonnes per year (Eftec 2022). They are typically emitted to sewers and become part of the wastewater which is treated in industrial and public wastewater treatment plants (WWTPs). In the European Union, the proportion of the population connected to wastewater treatment has increased steadily after the implementation of the Urban Wastewater Treatment Directive (EU 1991). In 2023, approximately 81% of the EU population was connected to at least secondary wastewater treatment. However, connection rates vary considerably across the European Union, ranging from 90–99% in some countries to less than 50% in others (Eurostat 2023).

Enzymes are water-soluble, non-adsorptive, non-volatile substances and remain in the aqueous phase unless degraded during wastewater treatment. Enzymes like subtilisin, α-amylase, cellulase,

and lipase are rapidly biodegradable as evidenced by results of OECD screening tests for ready biodegradability (HERA 2005, 2007). Certain enzymes in their active state, particularly subtilisins, may have adverse effects on aquatic organisms due to their biological activity. The no observed effect concentration (NOEC) in ecotoxicological studies is the highest concentration at which no adverse effects are observed. A very low NOEC of 6.0 µg active enzyme protein (aep) per litre (L) was reported for subtilisin in a test with early life stages of fish (*Danio rerio*) performed under flow-through conditions (Madsen et al. 2011). In comparison, α-amylase is less toxic to aquatic organisms, and a NOEC of 2600 µg aep/L was found in a test with the green alga *Desmodesmus subspicatus* (Madsen et al. 2011). Given both their propensity to be toxic to aquatic organisms and with the large amounts of enzymes entering wastewater systems, more information on the biotransformation and removal of enzymes in WWTPs is needed.

Although subtilisin and α-amylase are considered readily biodegradable, quantitative information on the disappearance of intact enzyme proteins under activated sludge conditions remains limited. There is a need to link laboratory-derived biotransformation rates with removal observed in full-scale wastewater treatment plants and to generate kinetic parameters suitable for environmental exposure assessment. The objective of this study was therefore to quantify the removal and primary biotransformation of subtilisin and α-amylase by combining monitoring data from an industrial and a municipal WWTP with laboratory activated sludge simulation tests.

In this study, biotransformation, also referred to as primary biodegradation, is defined as the microbially mediated chemical modification of intact enzyme proteins, leading to loss of detectable intact protein and biological activity. This process is distinct from ultimate biodegradation or mineralization to CO_2, although it may represent an initial step towards further biodegradation. Active enzyme proteins are intrinsically unstable molecules that undergo a variety of denaturation reactions when exposed to environmental conditions such as changes in temperature, chemicals, and catabolic enzymes produced by microorganisms (Iyer & Ananthanarayan 2008; Kumar et al. 2025). These

processes are particularly relevant when assessing the environmental exposure and persistence of enzymes released into wastewater systems.

MATERIALS AND METHODS

Enzymes

Commercial enzymes are identified by the Enzyme Commission (EC) number assigned by the International Union of Biochemistry and Molecular Biology (IUBMB) and by their numerical classification according to the European Inventory of Existing Commercial Chemical Substances (EINECS) and the Chemical Abstracts Service (CAS). The enzymes included in this study were subtilisin (EC 3.4.21.62, EINECS 232-752-2, CAS 9014-01-1) and α-amylase (EC 3.2.1.1, EINECS 232-565-6, CAS 9000-90-2). Samples of subtilisin and α-amylase for use in the laboratory studies were provided by Novozymes A/S, Bagsværd, Denmark. Both samples were received frozen and were kept in a freezer at -20°C until use. The sample of subtilisin (batch PPA40699) was produced by using a *Bacillus licheniformis* strain and was a brown liquid at room temperature with an enzyme concentration of 48.58 mg aep/g. The sample of α-amylase (batch PPY40206) was produced by using an *Aspergillus oryzae* strain and was a transparent yellow liquid at room temperature with an enzyme concentration of 93.89 mg aep/g.

Analytical methods

Sludge samples were stored at -20°C until the time of analysis in the laboratories at Novozymes A/S. The samples were thawed in a water bath (40°C) and then centrifuged at 1,000 × g for 5 min at 4 °C, while still cold. All samples were kept cold on ice before analysing the supernatants for either subtilisin or α-amylase.

Concentrations of subtilisin and α-amylase were determined using in-house sandwich enzyme-linked immunosorbent assays (ELISA), each specific to the respective target enzyme. Samples were analysed in duplicate, and absorbance was measured at dual wavelengths (490/620 nm). Enzyme concentrations were quantified against standard curves generated from dilution series (0.1-50 ng/mL) of the respective purified enzyme.

The ELISA standards containing subtilisin or α-amylase were prepared in supernatants from sludge to obtain the same matrix as that in the samples. The effect of the sludge matrix was minimal compared to standard ELISA buffer, and control samples were quantified equally well in both matrices. The loss of signal in the assay reflects disappearance of enzyme-specific epitopes.

Quality control procedures included duplicate measurements and inclusion of an assay control in each run, with an acceptance criterion of ±30%. The calibration curves showed strong linearity ($R^2 > 0.99$).

The limit of quantification (LOQ) was 0.2 µg aep/L for subtilisin and 0.3 µg aep/L for α-amylase in the analyses of enzymes in WWTPs, while it was 0.1 µg aep/L in the laboratory biotransformation experiments for both subtilisin and α-amylase.

Enzymes in wastewater treatment plants

The concentrations of intact subtilisin and α-amylase were measured by duplicate analyses of samples from the inlet and the outlet of an industrial and a municipal WWTP. The samples were kept cold in a thermos-box with cooling elements during transport for approx. 1 h and were stored at -20°C until they were sent frozen to the analytical laboratory for ELISA analysis of enzyme concentration.

KALUNDBORG is an industrial WWTP, located in Kalundborg, Denmark, which receives wastewater containing enzymes from production sites of Novo Nordisk and Novozymes A/S

(treated water is 5000-8000 m^3/d). Samples at KALUNDBORG (100 mL) were collected daily during two campaigns from 28th June to 5th July 2008, and from 6th to 16th July 2009, at the inlet to the primary separation tank and at the outlet.

LYNETTEN is a municipal WWTP, located in Copenhagen, Denmark, designed for treatment of wastewater from 1000000 person equivalents. Samples at LYNETTEN (100 mL) were collected daily between 8th and 13th June 2009 at the inlet, at the outlet from the primary separation tank, and at the outlet to the Øresund which is the strait between Denmark and Sweden.

Laboratory biotransformation experiments

Simulation test: Biotransformation experiments were conducted in the laboratory to determine the rates of transformation of intact subtilisin and α-amylase under aerobic conditions simulating activated sludge treatment. The experiments were based on measurements of the intact, active enzymes and were performed in accordance with the OECD 314B test method (OECD 2008). ELISA analyses were used to monitor the transformation (primary biodegradation) of intact subtilisin and α-amylase during 48 h in biotic sludge and abiotic sludge in which biological activity was inhibited.

Activated sludge: The activated sludge used in the biotransformation experiments was obtained from the aeration basin at the central municipal WWTP in Hillerød, Denmark, which receives wastewater from 65000 person equivalents. The temperature of the sludge at the time of collection was 13°C. During the transport to the laboratory for approx. 1 h, the temperature of the sludge increased to a maximum of 23°C. Upon receipt in the laboratory, the sludge was placed at 20 ± 2°C with continuous aeration. Following sieving through a 2-mm screen to remove coarse particles, the total suspended solids concentration in the sludge was approx. 5500 mg/L which was adjusted to approx. 3900 mg/L by dilution with tap water as described in the OECD 314B test method.

Biotic and abiotic sludge: Biotic sludge systems were prepared by distributing 1 L of the activated sludge into each of three replicate test flasks (4-L Erlenmeyer flasks). To reduce the effects of settling sludge particles, smaller volumes of approx. 0.2 L were added in a stepwise fashion across the test flasks. Abiotic sludge systems were prepared by autoclaving the activated sludge for at least 90 min at 121°C. The autoclaved sludge was added to three replicate test flasks using the same stepwise distribution method as for the biotic flasks, and sodium azide (NaN_3) was added to the abiotic test flasks at a final concentration of approx. 50 mM to inhibit the growth of bacteria surviving the autoclaving. Except for the autoclaving and the added bactericide, the biotic and abiotic sludges were equivalent to the extent possible.

Addition of test substance: Subtilisin and α-amylase were added to the test flasks from aqueous stock solutions prepared no more than 2 h before the initiation of the experiments by weighing the thawed, liquid enzyme sample into Milli-Q water that had been autoclaved for 20 min at 121°C. The stock solutions contained nominal concentrations of approximately 24 mg aep/L for both subtilisin and α-amylase.

Incubation: The biotransformation experiments were initiated one day after collecting the original sludge sample. The stock solutions of subtilisin and α-amylase were dosed gradually to the test flasks by using a pipette aiming at nominal concentrations of 50 µg aep/L. All test flasks were incubated in a room with a controlled temperature of 20 ± 2°C. The sludge in the biotic and abiotic test flasks was purged with sterile-filtered atmospheric air, and the test flasks were placed on a magnetic stirrer to keep the sludge in suspension and ensure that aerobic conditions were maintained. During the initial phase of frequent sampling, the test flasks were not shielded from the light in the room. After approx. 2 h, the test flasks were incubated in darkness for the remaining part of the experiment, except during sampling where the room light was turned on. The test flasks were closed with cotton stoppers to minimize loss of water by evaporation.

Oxygen and pH: Dissolved oxygen (O_2) and pH were measured before the initiation of the experiment and after the withdrawal of the last sample in all test flasks.

Samples for ELISA analysis: The first sludge samples were collected immediately after the addition of subtilisin or α-amylase to each test flask. A uniform procedure was used across all test flasks which implies that the first samples were taken approx. 0.25 min after dosing with enzyme. Subsequent samples were collected with short intervals during the next 6 h followed by sampling after 24 and 48 h of incubation. Samples of 3 mL were transferred to 15-mL polypropylene centrifuge tubes containing NaN_3 (equivalent to approx. 50 mM NaN_3 in 3 mL) to stop the biological activity. The sample tubes with the samples were immediately placed in a dry ice-ethanol bath. The samples were stored at -20°C until they were sent frozen to the analytical laboratory for ELISA analysis of enzyme concentration.

Kinetic analysis: The transformation of subtilisin and α-amylase was described by first order kinetics assuming that the concentration of the biomass did not change in the examined time window. Based on inspection of the disappearance of the intact enzymes during the first 5 min of the experiments, the data can be fitted to a two-compartment first order model (Federle & Itrich 2006):

$$[C] = [C]a \cdot e^{-k1t} + [C]b \cdot e^{-k2t},$$

where [C] equals the concentration remaining at time-5 min, [C]a equals the estimated initial concentration ($t_{0\text{-eff}}$) which is transformed at first order rate k1, and [C]b equals the concentration remaining at time-0.25 min which is transformed by first order rate k2. Thus, the two first order rate constants can be calculated by $k1 = \frac{-(\ln[C]b - \ln[C]a)}{t}$ and $k2 = \frac{-(\ln[C] - \ln[C]b)}{t}$.

When true first order kinetics exist, the biotransformation of a substance is often characterized by the half-life (T½) calculated by the equation T½ = ln2/k. The time for a reduction of the initial concentration of a chemical substance by 50% is known as Disappearance Time 50 (DT50), which

220 is a more appropriate descriptor when the data are too limited to verify true first order conditions
221 (OECD 2004, 2008). The estimated k1 constants in the present study were used to calculate the
222 DT50 by the equation DT50 = ln2/k. The term DT50 was preferred because the experiments were
223 performed with only one initial concentration of the test substances which does not permit a
224 detailed analysis of the degradation kinetics.

225

226 **RESULTS**

227 **Enzymes in wastewater treatment plants**

228 High concentrations of intact enzymes were detected in the inlet to KALUNDBORG as expected for
229 an industrial WWTP which receives wastewater from enzyme manufacturing processes. The
230 enzyme concentrations in the KALUNDBORG inlet ranged from approx. 300 to 65000 µg aep/L for
231 subtilisin and from approx. 30 to 6500 µg aep/L for α-amylase. The concentrations of the enzymes
232 in the KALUNDBORG outlet were below the LOQ of 0.2 µg aep/L for subtilisin and 0.3 µg aep/L for
233 α-amylase except for two samples in which the concentration of subtilisin was 0.4 aep/L (Table 1).
234 These results indicate that the influent subtilisin decreased by more than 99.9%, while the influent
235 α-amylase decreased by more than 99.0% during the treatment in KALUNDBORG.

236 At LYNETTEN, a predominantly municipal WWTP, influent concentrations were substantially lower.
237 Subtilisin concentrations ranged from <0.2 to 1.6 µg aep/L, while α-amylase concentrations ranged
238 from <0.3 to 0.4 µg aep/L. In the final effluent, both enzymes were below their respective LOQs
239 (Table 1).

240

241

242

Table 1 Measured concentrations of intact subtilisin and α-amylase in samples collected daily at the industrial wastewater treatment plant KALUNDBORG and the municipal wastewater treatment plant LYNETTEN. Enzyme concentrations measured in the collected number of samples (n) are shown as the range and, where applicable, the average ± standard deviation. The limit of quantification (LOQ) was 0.2 µg aep/L for subtilisin and 0.3 µg aep/L for α-amylase.

WWTP	**Sampling point**	**n**	**Subtilisin [µg aep/L]**	**α-Amylase [µg aep/L]**
KALUNDBORG (2008)	Inlet	8	286 - 38128 11737 ± 11467	Not analyzed
	Outlet	8	<0.2	Not analyzed
KALUNDBORG (2009)	Inlet	11	6093 - 65459 44604 ± 18396	30 - 6454 1242 ± 1936
	Outlet	11	<0.2 - 0.4	<0.3
LYNETTEN	Inlet	6	<0.2 - 1.6	<0.3 - 0.4
	After primary tank	6	<0.2 - 1.5	<0.3 - 0.4
	Outlet	6	<0.2	<0.3

Laboratory biotransformation experiments

Rapid biological inactivation of subtilisin and α-amylase was observed in the biotic activated sludge, and a true time zero concentration could therefore not be measured. The enzyme concentrations measured in the abiotic sludge after approx. 0.25 min were close to the calculated

253 nominal concentrations and were adopted as the effective time zero ($t_{0\text{-eff}}$) concentrations for the
254 biotic sludge system.

255 The measured enzyme concentrations demonstrating the disappearance of the intact subtilisin and
256 α-amylase during the experiments are shown in Table 2. The transformation of subtilisin and α-
257 amylase in the biotic sludge occurred in three phases:

258 (i) An initial phase of very rapid transformation within the first 0.25 min of the experiment during
259 which more than 70% of the intact enzyme protein was transformed.

260 (ii) A second phase of rapid transformation in the time from approx. 0.25 min ($t_{0.25\ min}$) to 5 min (t_{5}
261 $_{min}$) after the start of the experiment during which the loss of the intact enzyme exceeded 90%.

262 (iii) A terminal phase of continuous transformation in the time from 5 min until the end of the
263 experiment after 48 h during which the concentrations of the intact enzyme protein declined to a
264 value below the LOQ (0.1 µg aep/L).

265

Table 2 Measured concentrations of intact subtilisin and α-amylase in biotic and abiotic activated sludge during aerobic incubation at 20°C.

Time	Subtilisin [µg aep/L] [1]		α-Amylase [µg aep/L] [1]	
	Biotic sludge	Abiotic sludge	Biotic sludge	Abiotic sludge
$t_{0\text{-eff}}$	45 [2]	Not applicable	56 [2]	Not applicable
$t_{0.25\ min}$	8.2 ± 0.4	45 ± 4.2	16 ± 7.5	56 ± 14
$t_{1\ min}$	6.3 ± 1.3	41 ± 5.9	14 ± 1.2	49 ± 6.1
$t_{3\ min}$	4.5 ± 0.8	43 ± 7.0	7.7 ± 1.2	43 ± 2.3
$t_{5\ min}$	2.8 ± 0.4	39 ± 5.7	5.3 ± 2.5	45 ± 2.3
$t_{10\ min}$	1.7 ± 0.8	43 ± 6.4	2.3 ± 0.6	44 ± 4.0
$t_{20\ min}$	1.5 ± 0.2	45 ± 2.3	0.9 ± 0.1	45 ± 4.6
$t_{30\ min}$	1.2 ± 0.3	43 ± 9.2	0.5 ± 0.2	43 ± 6.1
$t_{60\ min}$	0.8 ± 0.0	34 ± 7.5	0.3 ± 0.2	45 ± 2.3
$t_{90\ min}$	0.5 ± 0.2	37 ± 4.0	0.15 ± 0.1	47 ± 4.6
$t_{2\ h}$	0.3 ± 0.1	35 ± 4.6	0.1 ± 0.0	43 ± 2.3
$t_{4\ h}$	0.3 ± 0.1	35 ± 6.4	<0.1	48 ± 0.0
$t_{48\ h}$	<0.1	31 ± 2.6	<0.1	47 ± 2.3

1) Averages of three replicates ± standard deviation

2) Derived from the measured concentration in the abiotic sludge after approx.0.25 min and used to represent the effective time zero ($t_{0\text{-eff}}$) concentration

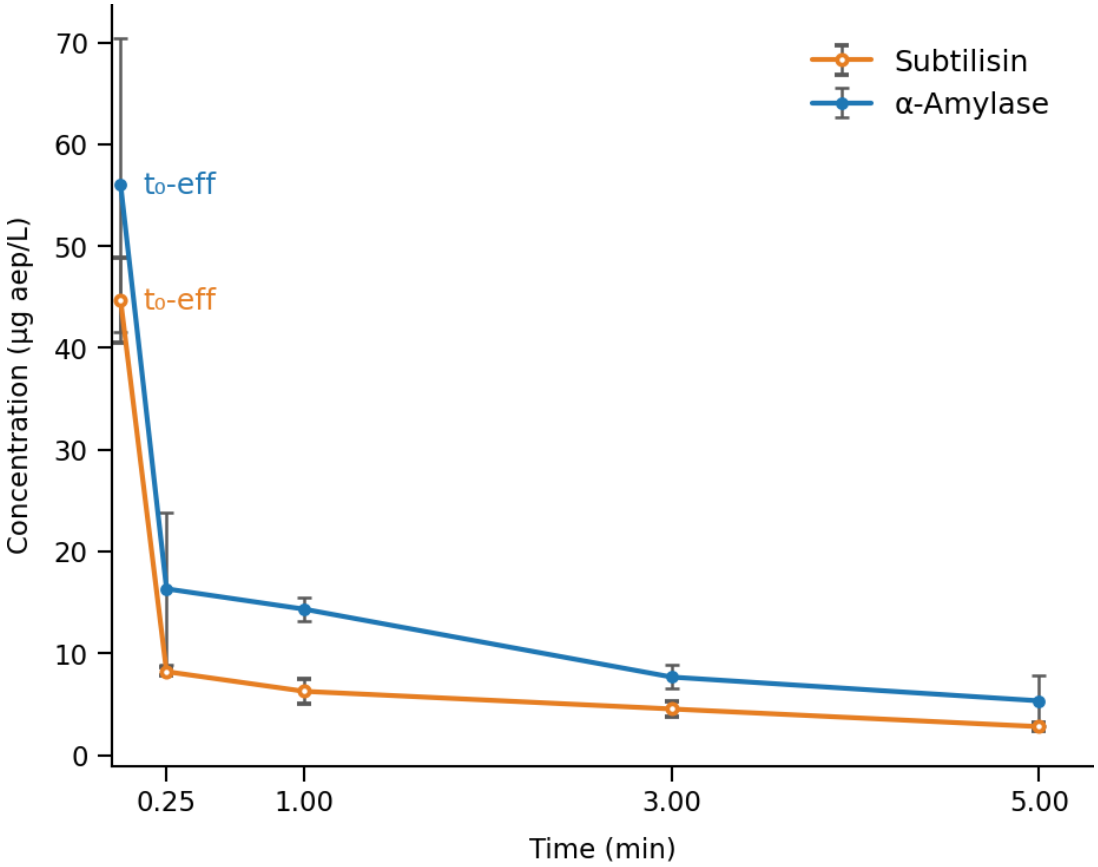

Figure 1: Biotransformation of subtilisin and α-amylase in biotic activated sludge during the first 5 min of incubation. Data represent mean concentrations (µg aep/L) of three replicates ± standard deviation. Lines represent first-order kinetic fits for the two transformation phases.

The rapid decrease of intact subtilisin and α-amylase during the first 5 min of the experiments is shown in Figure 1. The data in Table 2 and Figure 1 confirm that the transformation of the enzymes can be described by two distinct kinetic constants. Table 3 shows the calculated first order rate constants k1 describing the transformation in the first 0.25 min and k2 describing the transformation in the time from 0.25 min to 5 min of incubation. Graphs of the natural logarithm of the residual enzyme concentration versus time from 0.25 min to 5 min were linear with R^2 = 0.990 (subtilisin) and R^2 = 0.986 (α-amylase) supporting the assumption of a first order reaction.

Table 3 First order rate constants for the transformation of enzymes in biotic activated sludge.

Test substance	**Correlation**	**Enzyme concentration [µg aep/L] [1)]**		**Rate constants [min^{-1}] [3)]**
1st phase (0 to 0.25 min)	*R^2*	*Initial concn., $t_{0\text{-}eff}$ [2)]*	*Concn., $t_{0.25\ min}$*	*k1*
Subtilisin	1.00	45	8.2 ± 0.4	6.8
α-Amylase	1.00	56	16 ± 7.5	5.0
2nd phase (0.25 to 5 min)	*R^2*	*Concn., $t_{0.25\ min}$*	*Concn., $t_{5\ min}$*	*k2*
Subtilisin	0.957	8.2 ± 0.4	2.8 ± 0.4	0.17
α-Amylase	0.957	16 ± 7.5	5.3 ± 2.5	0.19

1) Averages of three replicates ± standard deviations
2) Derived from the measured concentration in the abiotic sludge after approx.0.25 min and used to represent the effective time zero ($t_{0\text{-}eff}$) concentration
3) Calculated from the equations $k1 = \frac{-(\ln[C]b - \ln[C]a)}{t}$ and $k2 = \frac{-(\ln[C] - \ln[C]b)}{t}$, where [C]a equals the estimated initial concentration ($t_{0\text{-}eff}$), [C]b equals the concentration remaining at time-0.25 min, and [C] equals the concentration remaining at time-5 min.

The rate constant k1 is the most relevant for predicting the removal of the intact enzymes in the activated sludge compartment of a WWTP. By using the k1 derived from the loss of the enzymes in the biotic activated sludge experiments, the Disappearance Time 50 (DT50) can be calculated to less than 10 seconds for both subtilisin and α-amylase. The concentrations of dissolved oxygen decreased to 63% (subtilisin) and 45% (α-amylase) in the biotic sludge during the experiment, while the dissolved oxygen remained at 98% after the end of incubation in the abiotic sludge. The pH was stable around 7.6-7.8 in the biotic sludge, while a decrease in pH from 8.5 to 8.1 was observed in the abiotic sludge.

DISCUSSION

Denaturation or unfolding of an enzyme protein is a process in which the native, intact structure of the protein is lost due to disruption of non-covalent interactions, leading to loss of enzymatic activity. A denatured or unfolded protein molecule is easily degraded by catabolic enzymes present in wastewater (Acharya & Chaudhuri 2021). The concentrations of subtilisin and α-amylase were below the LOQ in the outlet of the industrial WWTP, KALUNDBORG, which indicates considerable removal of more than 99.9% of intact subtilisin and more than 99.0% of intact α-amylase. Similarly, the treatment processes in the municipal WWTP, LYNETTEN, also led to a significant removal of subtilisin and α-amylase that reached concentrations below the LOQ in the outlet (Table 1). The low concentrations of intact enzymes in the inlet to LYNETTEN were expected, as substantial transformation and inactivation of enzymes occur during use and transport in the sewer system (HERA 2005, 2007).

Subtilisin and α-amylase were very rapidly transformed in the laboratory experiments with activated sludge, as more than 70% of the intact enzymes were lost during the first 0.25 min. Besides an active community of bacteria, activated sludge contains residuals of numerous substances including bacterial enzymes and enzymes used as ingredients in chemical products (e.g., detergents). The very rapid decline of subtilisin and α-amylase was probably due to microbially mediated transformation and reactions with catabolic enzymes in the activated sludge. Compared to the first 0.25 min of the experiment, the transformation of subtilisin and α-amylase continued with a slower, but still rapid rate, which was likely a result of the lower concentration of the intact enzymes. In the time from 0.25 min to 5 min, the transformation of the intact enzymes exceeded 90% of the initial concentrations (Table 2; Figure 1). After 5 min, the concentrations of intact subtilisin and α-amylase continued to decrease at much slower rates. This was likely due to the gradual depletion of subtilisin and α-amylase in the biotic sludge limiting the reactions with catabolic enzymes. In the abiotic sludge, a gradual and relatively slow decline of the intact enzymes was observed during the experiments (Table 2). The decrease of the intact enzyme

329 concentrations in the abiotic sludge may be due to adsorption of the enzyme to surfaces in the test
330 system or abiotic transformation.

331 Table 3 shows the first order rate constants for the transformation of subtilisin and α-amylase in
332 biotic activated sludge. The rapid biotransformation of the enzymes during the first 5 min was
333 expressed by the first order rate constants k1 (5.0 and 6.8 min^{-1} for α-amylase and subtilisin,
334 respectively) and k2 (0.17 and 0.19 min^{-1} for α-amylase and subtilisin, respectively). The k1
335 constants represent DT50 values of less than 10 seconds for α-amylase and subtilisin. Although
336 not demonstrated in the present study, the initial transformation is expected to lead to rapid
337 mineralisation of α-amylase and subtilisin as both enzymes are ultimately degraded in tests for
338 ready biodegradability (HERA 2005, 2007).

339 Recalling that subtilisin and α-amylase are used in detergent products, it is interesting that rapid
340 primary degradation of detergent ingredients like fatty alcohols and alcohol ethoxylates has also
341 been observed in simulation tests with activated sludge. With a similar rapid initial transformation
342 as that observed in the present study, the first order rate constants (k1) for the transformation of
343 the fatty alcohols and alcohol ethoxylates ranged between 1.4 and 2.4 min^{-1} corresponding to half-
344 lives of less than a minute (Federle & Itrich 2006).

345 Realistic biotransformation rate constants are essential for environmental exposure estimation and
346 for meeting regulatory requirements for chemical safety assessment. Measured transformation
347 rates from simulation tests are often not available, and in the absence of such data, substances
348 meeting the criteria for ready biodegradability (OECD 1992) can be assigned a default rate
349 constant (k) of 1 h^{-1} (ECHA 2026). The estimated first order rate constants for subtilisin and α-
350 amylase (Table 3) show that the default rate constant would be overly conservative for enzymes.
351 The results of the activated sludge simulation tests in the present study, and thus the rate
352 constants for subtilisin and α-amylase, were consistent with the complete removal of the enzymes
353 in real WWTPs (Table 1). Similarly, the rapid primary degradation of fatty alcohols and alcohol

ethoxylates in activated sludge simulation tests (Federle & Itrich 2006) was in good agreement with monitoring data confirming the removal of more than 99% of alcohol ethoxylates and other surfactants in WWTPs in the Netherlands (Matthijs et al. 1999). Activated sludge simulation tests can thus be used to predict the removal of biodegradable organic chemicals in WWTPs.

CONCLUSION

The present study quantified the removal and primary biotransformation of subtilisin and α-amylase by combining monitoring data from full-scale WWTPs with laboratory activated sludge simulation tests. In the industrial WWTP, removals exceeded 99.9% for intact subtilisin and 99.0% for intact α-amylase, while both enzymes were below their respective LOQs in the effluent of the municipal WWTP. In laboratory activated sludge simulation tests, intact subtilisin and α-amylase were transformed extremely rapidly with DT50 values of less than 10 seconds for both enzymes.

These results support the hypothesis that intact subtilisin and α-amylase are rapidly transformed in biotic activated sludge and that biotransformation explains the very low concentrations observed in WWTP effluents. Because the aquatic toxicity of enzymes is primarily associated with their intact and biologically active form, the rapid disappearance of detectable intact enzyme protein indicates that wastewater treatment substantially reduces potential aquatic exposure and associated ecotoxicological concern. The estimated first order rate constants and DT50 values provide experimentally based parameters that can be used to improve environmental exposure modelling and aquatic risk assessment of enzymes discharged to wastewater systems

Similar rapid transformation may be expected for other readily biodegradable enzymes, such as cellulases and lipases, although enzyme-specific confirmation would further strengthen this extrapolation.

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AUTHOR CONTRIBUTIONS

E. Bournaka and T. Madsen planned and performed the study and contributed to writing, reviewing, and editing the manuscript. M. Thorsen and B.M. Jensen planned and conducted the enzyme analyses and contributed to proofreading the manuscript.

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