ENZYME ACTIVITY OF WASTE ACTIVATED SLUDGE EXTRACTS

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ABSTRACT

Wastewater treatment and generated biological sludge provide an alternative source of enzymes to conventional industrial production methods. Here, we present a protocol for extracting enzymes from activated sludge using ultrasonication and surfactant treatment. Under optimum conditions, ultrasound disruption of activated sludge gave recovery rates of protease and cellulase enzymes equivalent to 63.1 % and ~100 %, respectively. The extracting enzymes from activated sludge represents a potentially significant, high value, resource recovery option for biological sludge generated by municipal wastewater treatment.

KEYWORDS

Activated sludge, cell disruption, enzymes, ultrasonication, resource recovery

INTRODUCTION

Enzymes are high value industrial biocatalysts with extensive applications in a wide range of manufacturing and processing sectors. The catalytic efficiency of enzymes can be several orders higher compared to inorganic chemical catalysts (e.g. metals, metal ions and metal oxides) under mild conditions (i.e. ambient temperature, atmospheric pressure and neutral pH) (Hermes et al., 1987). The global market of industrial enzymes has shown a steady increase since 1995 to $5.5 billion in 2018 and it is expected to reach $7.0 billion by 2023 at an annual growth rate of 4.9% (Arun, 2018). Among various enzymes, hydrolases (i.e. hydrolytic enzymes, such as proteases, lipases and amylases) are widely used in agriculture, food and the household care industries. However, the culture medium for enzyme production is a major reason for the high cost of industrial enzymes.

Activated sludge (AS) mainly consists of various microorganisms that are capable of degrading organic pollutants in wastewater by producing substantial quantities of hydrolytic enzymes. Thus, AS is potentially a cost-effective alternative raw material for hydrolytic enzyme

37 production. The enzymes are either adsorbed to the cell surface or embedded in the
38 extracellular polymeric substances (EPS) of the cellular biomass in AS. Enzyme extraction
39 following sludge floc disruption has been demonstrated and is technically feasible in bench-
40 scale experiments (Jung et al., 2002; Gessesse et al., 2003; Marin et al., 2018). Ultrasonication
41 is effective in destroying microbial cell membranes, releasing intracellular substances and
42 enzymes embedded in the sludge EPS matrix (Zielewicz, 2016) and is widely applied in
43 intracellular and/or extracellular extraction (Capelo et al., 2004; Hong et al., 2017). However,
44 the extraction efficiency is affected by the treatment conditions, including power intensity and
45 treatment duration. Triton X100 (TX100) is a non-ionic surfactant that is widely used in cell
46 lysis processes. It can permeabilize microbial cell membranes and improve the release of
47 cellular proteins (Koley and Bard, 2010).

48 No consistent, optimal approach is available for enzyme extraction process since the growth of
49 microorganisms and the production of bio-enzymes can be affected by a range of factors. Here,
50 we develop a technique for enzyme extraction from AS. Enzyme activity assays for four
51 different types of hydrolytic enzymes that are typically found in sludge were carried out,
52 including protease, amylase, cellulase and lipase. The performance of ultrasonication in
53 disrupting sludge flocs, applied in combination with TX100 surfactant, was examined. The
54 patterns of enzyme activity and the viability of the sludge biomass was also investigated for
55 the first time.

56

57 **METHODS**

58 **Sludge samples**

59 Sludge samples were collected from two major wastewater treatment plants in the UK,
60 WWTP1 and WWTP2, with treatment capacities of 180,000m^3/day and 53,000 m^3/day,
61 respectively.

62 WWTP1 operated a standard AS process and thickened waste activated sludge (WAS) samples
63 were collected from the thickening belts after flocculant dosing (Flopam, 0.24% w/w active)
64 at WWTP1.

65 Settled sewage was treated with a biological nutrient removal (BNR) process at WWTP2,
66 comprising anaerobic, anoxic and aerobic zones. Mixed liquor (ML) samples were collected
67 directly at five equidistant positions along the aeration tank, which is a plug flow reactor. The
68 mixed liquor suspended solids (MLSS) concentration at WWTP2 was typically in the range of
69 3800-4300mg/L. The ML samples were collected with a bucket and dosed with polyacrylamide
70 (Flopam, 0.24% w/w active) at a rate of 200 ml/20 L. The mixture was filtered after 30 minutes
71 through a strainer bag and the flocculated sludge was collected. A WAS sample was also
72 collected from the thickening belt, after polymer dosing.

73 Sludge samples were transported to the lab in an ice box on the day of collection and were
74 stored in a fridge overnight at 4 ^{o}C and enzyme extraction was performed the following day.

75 **Sludge Characterisation**

76 The total solids (TS) and volatile solids (VS) of the sludge samples were measured according
77 to standard methods (Eaton, 2005).

Protein and deoxyribonucleic (DNA) release provide indicators of cell lysis. The protein content of the sludge was determined by the Lowry Method (Lowry et al., 1951) using bovine serum albumin as the standard. The DNA content was quantified by the diphenylamine method (Li et al., 2014) using salmon sperm DNA as the standard.

All measurements were completed in triplicate.

Enzyme Activity Assays

Enzyme activity were measured based on the product formation during the hydrolysis reactions (Scopes, 2002; Bisswanger, 2011). One enzyme activity unit (U) was defined to generate 1 μmol of product per minute.

The enzyme activity assays were conducted in triplicate as follows:

1. *Protease activity* was measured using the Lowry method described by (Nabarlatz et al., 2010) with casein and L- tyrosine as the substrate and standard, respectively.
2. *α-Amylase activity* was determined by the 3, 5-dinitrosalicylic acid method with starch as the substrate and glucose as the standard, as described by Kanimozhi et al (2014). Pre-heating treatment at 68℃ was applied to deactivate β-amylase.
3. *Cellulase activity* was measured according to the carboxymethyl cellulose (CMC) method (Ghose, 1987) using CMC as the substrate and glucose the standard.
4. *Lipase activity* was determined using p-nitrophenol palmitate as substrate and the release of p-nitrophenol was measured by continuous spectrophotometric rate determination (Pencreach and Baratti, 1996).
5. *Dehydrogenase activity* was performed following the method of Yao et al. (2010), with 2,3,5-triphenyltetrazolium chloride as the substrate and triphenyl formazan formation was determined by toluene extraction.

Enzyme Extraction Protocol

The sludge was centrifuged at 2000g for 15 min to remove excess water content, and the sediment was washed with 10mM Tris-HCl (pH 7.0) buffer and centrifuged again at 5000g for 15 min. The sediment was collected and re-suspended in 10mM Tris-HCl buffer (pH 7.0) to its original volume. The suspension was diluted with buffer and subjected to ultrasonic disruption (VCX130, Sonics & Materials, Inc., UK) for a certain time period (as prescribed by the experimental design – see later), after which the suspension was shaken at 120 rpm for 45 min and centrifuged at 12000g for 15 min. The supernatant was collected as crude enzyme extract.

To prevent warming of the samples and to preserve biological activity in the extract, ultrasonic disruption was carried out in an ice-water bath and the ultrasound pulse was set to 10 seconds on and 10 seconds off; other operations including centrifugation and washing of the sludge were carried out at 4°C.

Optimisation of Enzyme Extraction Protocol

Thickened sludge samples from WWTP1 were used for the optimisation process.

Effect of Ultrasonication Conditions

The operational parameters of ultrasonication include treatment duration and energy intensity. Sludge samples were diluted with 10mM Tris-HCl (pH 7.0) buffer to a ratio of 1:5 of the

118 original VS content before ultrasound disruption; the final solids concentration was 10.6g
119 VS/L.

120 The sonicator and probe delivered a constant amplitude (AMP) with a corresponding power
121 input of 76 micrometres at 100% AMP. To investigate the impact of treatment duration, the
122 diluted sludge samples were subjected to ultrasonication at 40% AMP for 2, 5, 10 and 15 min.
123 The impact of energy intensity was examined by disrupting diluted sludge samples (a dilution
124 factor of 5 was applied) at 20%, 40%, 60% and 80% AMP for 10 min; the corresponding energy
125 intensities during treatment were 343, 872, 1547 and 2312 W/L.

126 *Effect of Solids Content*

127 The sludge sample had a solids content of 69.4±0.42 g TS/L and 53.2±0.10 g VS/L and was
128 diluted with 10mM Tris-HCl buffer to provide a dilution factor (DF) of 2, 3, 5 and 10 before
129 ultrasonication. The sludge disruption was performed at 40% AMP for 10min. The specific
130 energy input was calculated by the following equation and is shown in Table 1.

131
$$\text{Specific energy input (kJ/g VS)} = \frac{\text{Total energy input per sample (kJ)}}{\text{Volatile solids content (g VS/L)} \times \text{Sample volume (L)}}$$

132 **Table 1 Specific energy input by the ultrasonicator at different dilution factors for sludge**
133 **samples**

Dilution factor	2	3	5	10
Specific energy input (kJ/g VS)	21.8	30.4	49.9	99.2

134
135 *Effect of Surfactant Addition*

136 Sludge samples were diluted (DF=5) with 10mM Tris-HCl (pH 7.0) buffer (containing 1% v/v
137 TX100) and subjected to ultrasound disruption at 40% AMP and 10min duration.

138 *Patterns in Sludge Microbial and Enzymic Activities*

139 Mixed liquor sludge samples from the aeration tank, and WAS samples from the thickening
140 belt, at WWTP2 were used to investigate the patterns of enzyme activity and the viability of
141 the sludge biomass.

142 Sludge samples were diluted (DF=5) before ultrasonic disruption (40% AMP for 10 min, 1%
143 v/v TX100 addition). Dehydrogenase activity was used as an indicator of the general rate of
144 microbial activity.

145 **Enzyme Recovery Rate**

146 The sludge sample from WWTP1 was spiked separately with commercial protease and
147 cellulase enzyme products and was subjected to the same extraction protocol under the
148 optimum operational conditions, to determine the enzyme recovery rate. The recovery rate (*R*)
149 was calculated as follows:

150 $$R=\frac{Total\ enzyme\ activity\text{-}Background\ enzyme\ activity}{Spiked\ enzyme\ \ activity}\times 100\%$$

151 Where,

152 *Background enzyme activity* refers to the activity of the crude products extracted from sludge
153 samples without enzyme spike, and *Spiked enzyme activity* was obtained from standard enzyme
154 profiles (data not shown).

155

156 **RESULTS AND DISCUSSION**

157 **Enzyme Extraction Efficiency**

158 *Effect of Ultrasonication Conditions*

159 Ultrasonication conditions had a profound influence on the enzyme extraction efficiency.

160 Figure 1 shows the effect of different durations of ultrasonication on protein and DNA release,
161 which provide markers of the effects of the treatment on cell disruption. Protein and DNA
162 release increased with duration, but the magnitude of the response generally declined with
163 increasing treatment time. A similar response was also observed with the enzyme recovery
164 patterns, with maximum enzyme activities being observed at 15min duration, shown in Figure
165 2. α-Amylase gave the largest overall enzyme activity in AS, followed by cellulase and
166 protease, with maximum enzyme activity units per g VS of approximately 25, 7.5 and 3.0,
167 respectively.

168 The ultrasonicator used in this work was AMP controlled, thus, under certain amplitudes, the
169 energy consumption was directly proportional to the processing time. Extending the processing
170 time from 10 to 15 min, increased the energy consumption also by 1/3 (data not shown).
171 However, the activities of protease, α-amylase and cellulase were only modestly improved with
172 increasing energy input, by approximately 10%, 17% and 9.0%, respectively. Therefore, 10
173 min was selected as the optimum duration for ultrasonication treatment, which also offered
174 practical advantages compared to the longer duration period, by reducing the sample processing
175 time and the risk of heating the sample and enzyme denaturation. The activities of protease, α-
176 amylase and cellulase obtained were equivalent to 2.7, 22.4 and 6.8 U/g VS, respectively.

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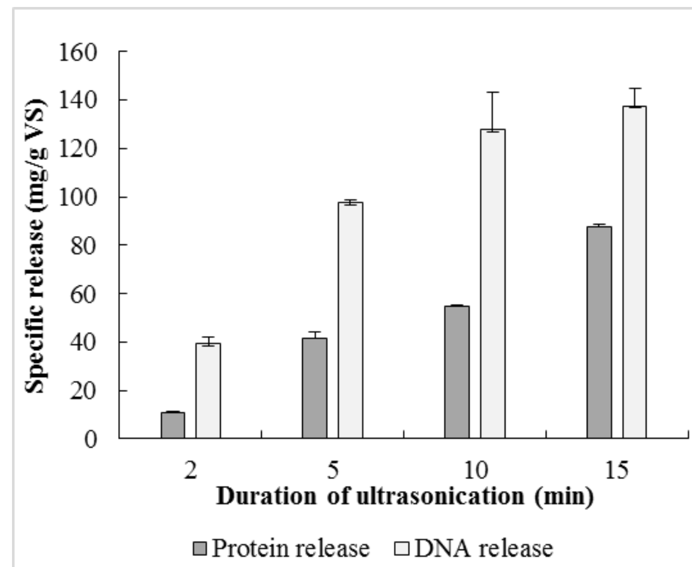

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179 **Figure 1 Effect of ultrasonication duration (minutes) on protein and DNA release from**
180 **activated sludge at 40% amplitude (vertical bars represent the standard deviation, n=3)**

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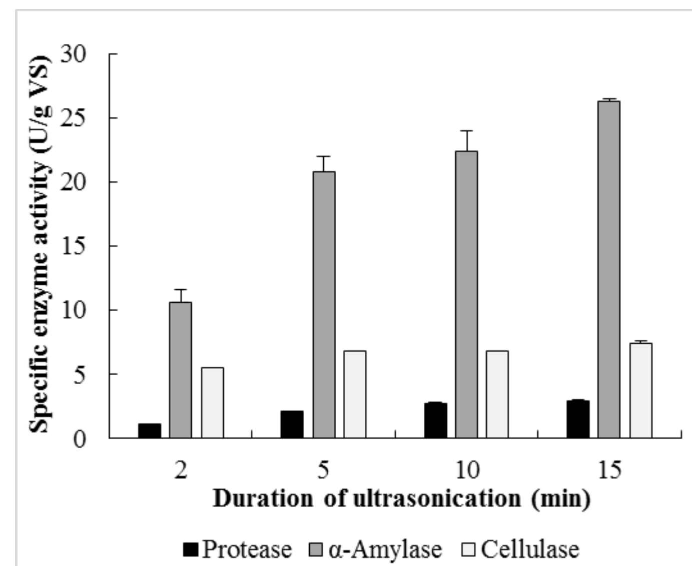


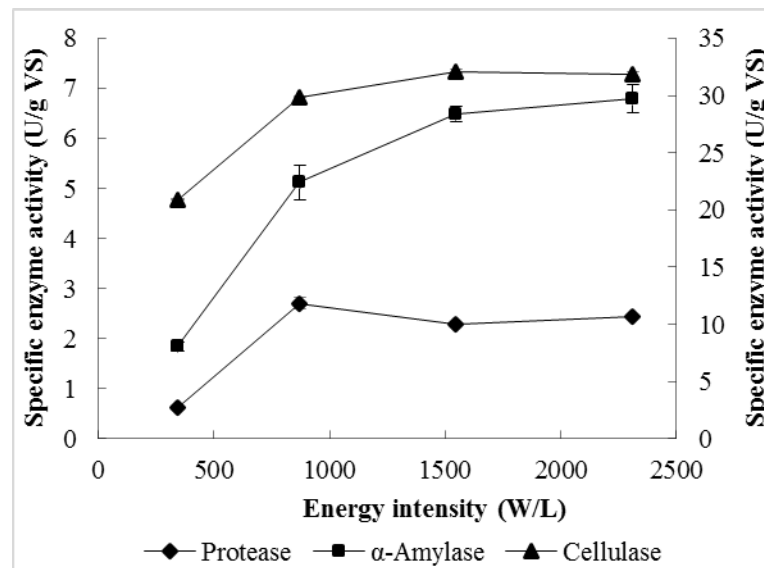
182

183 **Figure 2 Effect of ultrasonication duration (minutes) on enzyme activity at 40% amplitude**
184 **(vertical bars represent the standard deviation, n=3)**

185

186 Figure 3 shows the extraction efficiency of sludge sonication treatment at different AMP and
187 10 min duration; increasing the AMP raises the energy intensity. The specific enzyme activity
188 increased with energy intensity and the maximum activity of protease, α-amylase and cellulase
189 was 2.71 U/g VS at 872 W/L (40% AMP), 29.7 U/g VS at 2312 W/L (80% AMP) and 7.34
190 U/g VS at 1547 W/L (60% AMP), respectively. Amplitudes of 60% and 80% caused rapid
191 heating of the samples, despite the measures adopted to control the sample temperature by
192 completing the cellular disruption step in an iced-water bath. Reduced thermostability and

193 damage to the chemical bonds that maintain enzyme structural conformation may account for
194 the reduced activity of AS protease enzyme at the higher energy intensities (Nadar and Rathod,
195 2017), compared to α-amylase and cellulase (Figure 3). Therefore, 40% AMP (energy intensity
196 = 872W/L) was selected as the optimum energy intensity level for further experiments.



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198 **Figure 3 Effect of ultrasonication energy intensity (for 10 min duration) on enzyme activity;**
199 **activity of protease and cellulase are shown on the left axis and α-amylase is shown on the right**
200 **axis (vertical bars represent the standard deviation, n=3)**

201

202 The extracellular enzymes of bacteria are either accumulated in the gel-like EPS matrix, which
203 has a three-dimensional structure with an extremely large surface area that holds microbial
204 cells together to form the sludge floc, or tightly bound to cell membranes via hydrogen and/or
205 ionic bonding (Yu et al., 2007; Lin et al., 2014). A possible hypothesis about the mechanism
206 of enzyme release from AS by ultrasound treatment is that the cavitation generated by
207 ultrasound disrupts the EPS matrix that act as a protection layer of enzymes, after which the
208 enzymes in EPS are exposed to the surrounding aqueous solution and are readily detached by
209 shear forces induced through shaking within the solution (Wingender et al., 1999; Karn et al.,
210 2013; Nadar and Rathod, 2017). Cavitation generated by ultrasound also produces pores on the
211 cell membrane, releasing periplasmic enzymes and cellular proteins (Loustau et al., 2018). In
212 this study, DNA, which is mainly located in the nucleoid of bacteria, was detected in the crude
213 enzyme extract, indicating that the disruption of cell membranes had occurred. Consistent
214 exposure to cavitation, which, in the case of this study, was provided through extended
215 ultrasound treatment time, causes more severe damage of the weakened cell membrane and the
216 release of intracellular substances to solution.

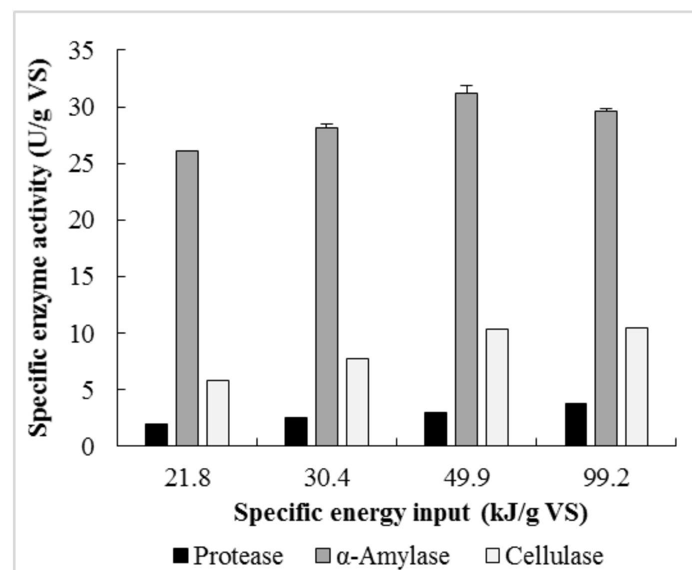
217 The results from the ultrasonication extraction performance analysis showed that short duration
218 or low amplitude (corresponding to low energy intensity) are were not effective at cellular
219 disruption and enzyme release. Therefore, it is necessary to provide suitable conditions that
220 achieve the "lysis threshold" when using ultrasound for cell disruption, and this is specific to
221 the type of cells under investigation (Rubin et al., 2018). Our results were consistent with
222 results by Zhang et al. (2007) and demonstrated effective AS cell lysis after 10 min
223 ultrasonication. Monique et al. (2008) found that protein release increased with ultrasound
224 treatment time, from 100 mg protein/g VSS after 2 min to 160 mg protein/g VSS after 10 min.

225 Hong et al. (2017) also found enhanced cell lysis, and greater EPS release, occurred with higher
226 ultrasound power intensities and longer treatment times.

227 *Effect of Solids Content*

228 The fundamental principle of sonication treatment is the conversion of electrical energy to
229 mechanical vibrations. Extensive micro-bubbles (cavities) are produced, which expand and
230 implode violently within a certain dispersion radius, generating extremely high pressures that
231 destroy microbial cells. Consequently, the ultrasonication probe has an effective range over
232 which the mechanical energy is gradually consumed, depending on the operational conditions
233 and solids content. Therefore, the optimum solids content to ensure efficient cellular disruption
234 of the sludge sample is necessary to maximise enzyme release per unit mass of sludge.

235 Figure 4 shows the enzyme activities of protease, α-amylase and cellulase with increasing
236 sludge dilution; the maximum activities were obtained at DF=5 and were equivalent to
237 approximately 3.0, 31 and 10.4 U/g VS activity, respectively. Increasing the DF from 5 to 10
238 almost doubled the energy input from 49.9 kJ/g VS to 99.2 kJ/g VS (see Table 1) but had no
239 effect on enzyme activity. Therefore, the solids content of sludge samples for sonication were
240 prepared at the optimum DF=5 (approximately 10g VS/L) in subsequent experiments.



241

242 **Figure 4 Effect of solids content on enzyme activity after ultrasonication treatment for 10 min**
243 **duration and 40% amplitude (vertical bars represent the standard deviation, n=3)**

244

245 *Effect of Surfactant Addition*

246 TX100 increased the release of extracellular proteins, which would be mainly associated with
247 the EPS fraction of the sludge flocs, and more than twice the amount of protein was measured
248 with TX100 compared to the control treatment without surfactant addition (Table 2). However,
249 no effect of TX100 was observed on DNA release or cell lysis. This behaviour was consistent
250 with results reported by Glauche et al. (2017) showing that the addition of TX-100 (2% v/v)
251 for cell disruption of *E.coli* maximised the concentration of soluble proteins.

252 The results from the surfactant experiments (Table 2) showed that the addition of 1% (v/v)
253 TX100 significantly improved protease release, almost doubling the activity of this enzyme.
254 Relative to protease, surfactant addition had a comparatively smaller effect on the activity of
255 α-amylase and cellulase. The effect of TX100 addition on the enzymic activity of AS extracts
256 may be explained by the cellular distribution of the enzymes. For example, according to Yu et
257 al. (2007), the majority of protease enzymes were found attached to the cell wall, whilst
258 amylase and other, related, sugar-degrading enzymes were mainly found in loosely bound-EPS
259 (LB-EPS). Therefore, surfactant treatment increases protease extraction by removing the
260 protective EPS layer. By contrast, amylase and cellulase are present in LB-EPS and are readily
261 extracted directly without TX addition.

262 **Table 2 Effect of surfactant addition on sludge disruption and enzyme activity**

	Protease (U/g VS)	α-Amylase (U/g VS)	Cellulase (U/g VS)	Protein release (mg/g VS)	DNA release (mg/g VS)
Without TX	2.71±0.11	22.39±1.53	6.83±0.02	54.70±0.42	127.63±15.57
With 1% TX	5.62±0.04	24.64±0.59	7.72±0.25	128.66±8.22	126.43±8.82
Improvement	+107.6%	+10.0%	+13.1%	+135.2%	-0.94%

263

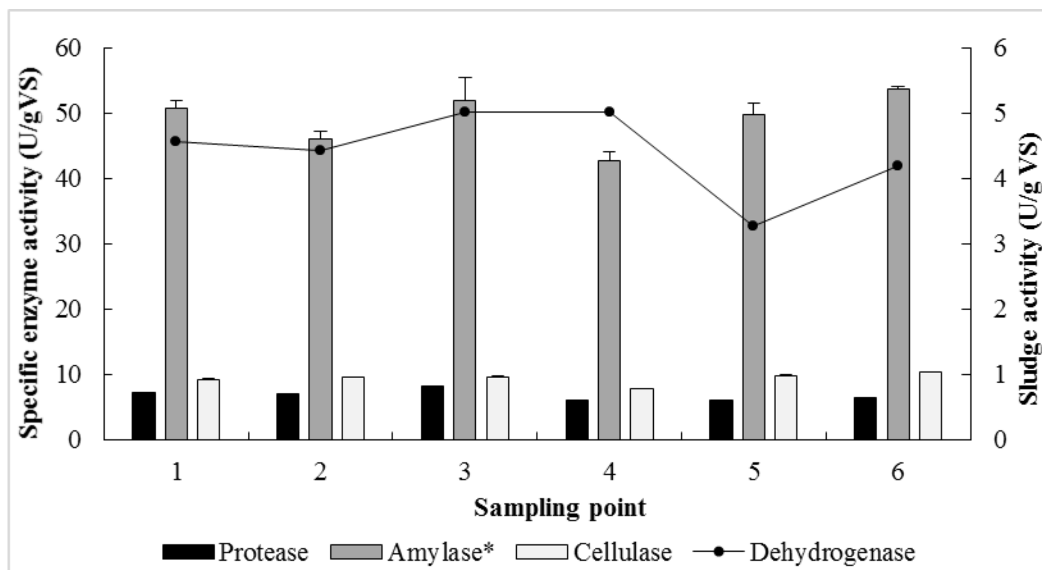
264 **Patterns in Sludge Microbial and Enzymic Activities**

265 Specific enzyme activities measured at the different sampling points of the AS aeration tank at
266 WWTP2 are shown in Figure 5. Sludge microbial activity, was determined based on
267 dehydrogenase activity, and increased from the inlet of the aeration tank to the centre position
268 (Point 3), and the maximum rate was equivalent to 5 U/g VS. As may be expected, the rate of
269 microbial activity decreased at the end of the aeration tank, by approximately 35%, reflecting
270 substrate exhaustion. Dehydrogenase is an oxidoreductase that plays an important role in
271 catalysing bio-chemical reactions for microbial dissimilation. It is involved in transferring
272 electrons from the substrate to an electron acceptor, usually NAD+/NADP+, in the cell. The
273 enzyme activity is indicative of the rate of electron transport when active microorganisms
274 oxidise organic pollutants in wastewater, and there is a strong correlation between
275 dehydrogenase activity and microbial oxygen uptake rate (OUR) (Awong et al., 1985; Bohacz,
276 2018). Thus, higher OUR indicates higher rates of microbiological activity. Consequently,
277 dehydrogenase is frequently used for measuring, and provides an effective indicator of, the
278 activity of the microbial biomass in AS (Goel et al., 1998; Feng et al., 2016; Robledo-Mahon
279 et al., 2019). Indeed, the results reported here were consistent with the microbial growth
280 observed in plug flow, aerobic biological sewage treatment reactors (Tchobanoglous et al.,
281 2014), where returned AS is activated when combined with the incoming substrate stream in
282 settled sewage, and reduced microbial activity and growth rates are observed due to
283 endogenous respiration as substrates are exhausted.

284 By contrast, hydrolytic enzyme activities in the AS extract did not follow the same patterns
285 observed in dehydrogenase activity and were generally relatively consistent at the different
286 sampling locations and there was no statistically significant correlation between them (*P* values
287 of Pearson correlations for protease, amylase and cellulase activity relative to dehydrogenase
288 activity were 0.34, 0.60 and 0.26, respectively). The maximum activities of the extracted
289 enzymes from the aeration tank were: 8.2 U/g VS, 52.2 U/g VS and 9.9 U/g VS for protease,
290 amylase and cellulase, respectively, which were also comparable to those observed for sludge

291 sampled from the thickening belt (Point 6). The apparent maintenance of hydrolytic enzyme
292 activities even under conditions of severe nutrient deprivation may be related to a microbial
293 ecological strategy to survive extreme conditions. Indeed, Kovárová-Kovar and Egli (1998)
294 showed that hydrolytic enzymes involved in bacteria carbon catabolism are active, not only
295 when organic substrates are sufficient at the inlet to the AS process, but also when organic
296 carbon sources are not available. The enzymes are maintained within the sludge flocs, mainly
297 in EPS fraction of the cell, and allow the hydrolysis and rapid assimilation of new substrates
298 when they become available in the surrounding environment, without the need to divert
299 resources to enzyme synthesis.

300 From a practical perspective, the results also demonstrated that thickened WAS was a suitable
301 source of biomass for enzyme extraction recovery that was easy to access and collect and had
302 a comparable enzyme activity to mixed liquor from the aeration tank.



303

304 **Figure 5 Enzyme and sludge microbial activity (as dehydrogenase activity) at different sampling**
305 **points along the length of an activated sludge aeration reactor; sample point 6 was taken from**
306 **the activated sludge thickening belt (vertical bars represent the standard deviation, n=3)**

307 *Note: preheat treatment was omitted from the enzyme assay and total amylase activity is
308 reported (including α- and β-amylase)

309

310 **Enzyme Recovery Rate**

311 The activities of four different hydrolytic enzymes (protease, lipase, amylase and cellulase),
312 representing the enzymes responsible for hydrolyzing the main organic constituents in urban
313 wastewater (protein, fat, starch and cellulose, respectively) were measured in AS samples
314 collected from the thickening belt at WWTP1 following the optimum ultrasonication extraction
315 protocol (40% AMP, 10 min duration, DF=5, 1% v/v TX100 addition) (Table 3). The activities
316 of the extracted enzymes were similar between different batches of sludge (data not shown),
317 indicating the developed protocol for enzyme extraction was consistent and not influenced by
318 variations in treatment conditions at the WWTP.

319 **Table 3 Average activities of major hydrolytic enzymes extracted from activated sludge under**
320 **optimal conditions (n=3)**

	Protease	Lipase	Amylase*	Cellulase
Specific activity (U/g VS)	8.40±0.19	21.72±1.88	39.4±0.31	13.48±0.08

321 *Note: preheat treatment was omitted from the enzyme assay and total amylase activity is
322 reported (including α- and β-amylase).

323

324 Protease and cellulase were selected to examine the recovery rate of enzymes from sludge using
325 the proposed protocol. The recovery rate was 63.1% and 115.3% for protease and cellulase,
326 respectively. Similar recovery rates for protease have been reported. For example, Ni et al.
327 (2017) obtained a recovery rate of 66.7% by stirring sludge with TX 100 for 60 min.
328 Interestingly, the activity of cellulase in the sludge extract was apparently increased with the
329 addition of commercial enzyme (see Methods section), thus a recovery rate >100% was given
330 by the activity assay. Such hyperactivation of enzymes may be explained by conformational
331 changes of the enzyme structure caused by ultrasonication under certain circumstance:
332 ultrasound treatment can cause the breakage of covalent bonds within the enzyme (Ladole et
333 al., 2017), releasing more active sites from inside the protein structure, resulting in higher
334 apparent enzymatic activity when exposed to the substrate (CMC in this case). Ladole et al.
335 (2017) found the hyperactivation for cellulase by ultrasonication was linked to significant
336 changes in the α-helix and β-sheet ratio within the secondary protein structure of the enzyme.
337 Ultrasound treatment can also improve activity through increased mixing and diffusion of the
338 reactive components of both substrate and enzyme (Capelo et al., 2004).

339

340 **CONCLUSIONS**

341 Ultrasonication was effective at disrupting AS flocs and releasing hydrolytic enzymes and the
342 results demonstrated that the developed protocol was a suitable approach for extracting
343 enzymes from WAS. The optimum operational parameters were: 40% AMP (equivalent to an
344 energy intensity of 872W/L) and 10 min duration. The solids content of sludge samples was
345 also an important parameter influencing ultrasonic disruption and optimisation experiments
346 indicated that a solids content of approximately 10g VS/L (DF=5) provided the maximum
347 enzyme activity. Surfactant addition (1% v/v TX100) enhanced protein release as well as
348 enzyme activity in the AS extract. Under optimum conditions, the recovery rates of protease
349 and cellulase were 63.1% and ~100%, respectively.

350 No correlation was found between sludge microbial activity and the activity of hydrolytic
351 enzymes. Therefore, thickened WAS, collected directly following secondary clarification, is a
352 viable and practical source of biomass for enzyme extraction.

353 Hydrolytic enzymes play a significant role not only in the Water Industry (e.g. pre-treatment
354 of wastewater and sludge digestion), but also in other industries (e.g. agriculture and house-
355 care). Therefore, future work will focus on the purification and concentration of hydrolytic
356 enzymes from AS to improve the industrial utility and applications of the enzyme products.

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