Electronic Supplementary Material (ESI) for Analytical Methods.

Supplementary Material

An improved, optimised and robust keratin azure assay for accurate assessment of keratinase activity.

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Context

Abbreviations

SOP	Standard operating procedure
PK	Proteinase K
SC	Subtilisin Carlsberg
KA	Keratin Azure
RBBR	Remazol Brilliant Blue R dye
TCEP	tris(2-carboxyethyl)phosphine
DF	Dilution factor
LOD	Limit of detection
LOQ	Limit of quantification
TCA	Trichloroacetic acid treatment
UP	Unprocessed
AC	Autoclaved
M	Milled
W1	Washed once
W3	Washed three times
NW	No work up
F	Filtration
C	Centrifugation
I	Iced

Keratin Azure Assay

SOP

Background:

This assay is used to detect keratinase activity on α-keratin. The standard assay uses keratin azure as the substrate which is calibrated with Remazol Brilliant Blue R Dye. The commercial proteinase K is used as a positive control.

Aim:

Detection and quantification of keratinase activity of novel or known keratinases on α-keratin.

Materials:

The materials, equipment and software described below are those used in the optimisation of this assay and are recommended. Alternative providers are available, however, the effects of different providers materials and/or instruments could have on this method have not been investigated.

Consumables:

- Keratin Azure (KA, Sigma-Aldrich – K8500)
- Proteinase K from *Tritirachium album* (PK, Sigma-Aldrich – P2308)
- Ramezol Brilliant Blue R Dye (RBBR, Thermofisher – 207550250)
- Tris-HCl (Trizma® hydrochloride, Sigma-Aldrich – T3253)
- Eppendorfs 1.5 mL
- 96-well plate

Equipment:

- Ball Mill (Retsch MM400 mill, Retsch MM400 25 mL stainless steel grinding jars and one 1.4 cm Ø grinding ball)
- Balance to ±0.0001 g
- Pipettes (P1000, P200)
- Orbital Mixer (Stuart Scientific, blood tube rotator SB1)
- Incubator
- Benchtop centrifuge (Thermo Scientific, Heraeus Pico 17)
- Plate Reader (BioTek Synergy HT)

Software:

- Origin 2019 version 9.6.0.172
- Excel 2016
- Gen 5 version 2.00.18

Plate Reader Method:

Table A. Readings performed in kinetic mode.

Instrument Condition	Setting
Plate	96-well plate
Setting	Runtime
Temperature	22 °C (pre-heat if required)
Shake	Medium, 5 s at start
Kinetic runtime	10 min
Reading interval	1 min
Total no. of readings	11
Wavelength	595 nm

Calibration Method:

1. Weigh 12.53 mg of RBBR standard.
2. Make up to 20 mL with appropriate buffer (PK buffer: 50 mM Tris-HCl, pH 9.0), making a 1 mM stock.
3. Preform, in triplicate, serial dilutions with buffer to make a range of concentrations between 500.00 – 1.95 μM.
4. Pipette 200 μL into a 96-well plate and measure the absorbance using the plate reader method, **Table A**.
5. Process the raw data by applying any path length corrections required, **Table B, Step 1**.
6. Take the mean and subtract the blank calculate the standard deviation for each concentration, **Table B, Step 2**.
7. Plot the readings in origin, **Figure A**.
8. Create a line of best fit to get the equation of the line, **Figure A** calibration curve.

NOTE: Each plate reader may have a different path length. Ensure this is taken into account in the adjustment of the absorbance readings. See equation below tables for examples.

Calibration Example

Table B. Sample data for the 62.5 μM RBBR standard in 50 mM Tis HCl pH 8.0. Step 1 (light blue): path length correction (0.64 cm). Step 2 (blue): calculate the mean and standard deviation. * Blank was also path length corrected to 0.0542.

Time (min)	Raw Data (Abs at 595 nm)			Path length correction (Abs/0.64 cm)		
	1	2	3	1	2	3
0	0.382	0.378	0.382	0.597	0.591	0.597
1	0.384	0.378	0.383	0.600	0.591	0.598
2	0.386	0.378	0.380	0.603	0.591	0.594
3	0.384	0.377	0.380	0.600	0.589	0.594
4	0.384	0.377	0.380	0.600	0.589	0.594
5	0.384	0.375	0.379	0.600	0.586	0.592
6	0.383	0.374	0.379	0.598	0.584	0.592
7	0.383	0.374	0.378	0.598	0.584	0.591
8	0.383	0.374	0.377	0.598	0.584	0.589
9	0.381	0.373	0.377	0.595	0.583	0.589
10	0.380	0.372	0.376	0.594	0.581	0.588
				mean		0.593
				mean - blank*		0.538
				std dev		0.006

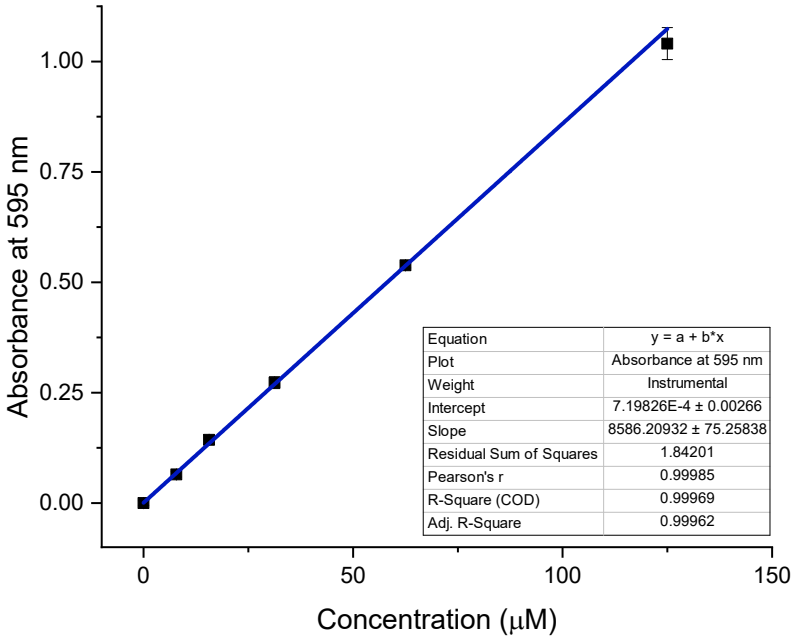

Figure A. Example Calibration Curve. Created in Origin.

Assay Method:

1. Mill ≤ 10 g of KA (30 Hz/s for 3 min).
2. Weigh 0.005g of milled KA ±0.0005g into a 1.5 mL eppendorf. See **Table C** for the amount of samples per enzyme.
3. Wash each KA sample with 1 mL deionised water. Invert to ensure KA is washed thoroughly. Centrifuge for 10 s and then remove liquid
4. Repeat the wash for a total of three washes.
5. Make the enzyme stocks (e.g. PK 50 μM stock: 7.2 mg into 5 mL of buffer)
6. Follow the volumes in **Table C** to add the buffer and then enzyme.

NOTE: When adding buffer, invert to ensure there are no bubbles and that the KA is not clumped at the bottom.

Component	Reaction	Positive Control (PK)	Negative Control (no enzyme)	Negative Control (no KA)	Blank (buffer only)
Replicates	3	3	3	3	3
KA	0.005 g	0.005 g	0.005 g	-	-
Enzyme	50 μL	50 μL (PK)	-	50 μL	-
Buffer	450 μL	450 μL	500 μL	450 μL	500 μL

Table C. Reaction mixtures.

7. Put on mixer at 35 rpm (fixed rotation speed) for 3 hrs.
8. Take samples and centrifuge for 10 min at 13 kRPM.
9. Without disturbing the pellet, pipette 200 μL of the liquid sample into a well on a 96-well plate.
10. Read the plate by following the plate reader method.
11. Preform a 2x dilution with buffer for every reading above 1.0 Abs.
12. Process the raw data by applying any path length corrections required (**Table D, Step 1**).
13. Subtract blank from each replicate (**Table D, Step 2**).
14. Use the calibration equation to determine the concentration. **Table D, Step 3**.
15. Adjust the reading for any dilutions.
16. Take the mean and standard deviation of the replicates.
17. Plot if required.

Table D. Sample data, SC Unprocessed. Step 1 (light blue): path length correction (0.64 cm). Step 2 (blue): calculate the mean and subtract the blank value. Step 3 (light green): use the standard curve to calculate the concentration (y=8638.870x-0.002), include the dilution factor and adjust the units for concentration or rate. Step 4 (green): calculate the mean and standard deviation.* Blank was also path length corrected to 0.0671.

	Raw Data (Abs at 595 nm)			Path length correction (Abs/0.64 cm)		
Time (min)	1	2	3	1	2	3
0	0.121	0.270	0.456	0.189	0.422	0.713
1	0.121	0.271	0.457	0.189	0.423	0.714
2	0.121	0.272	0.458	0.189	0.425	0.716
3	0.121	0.273	0.458	0.189	0.427	0.716
4	0.121	0.273	0.458	0.189	0.427	0.716
5	0.121	0.273	0.458	0.189	0.427	0.716
6	0.121	0.274	0.458	0.189	0.428	0.716
7	0.120	0.273	0.458	0.188	0.427	0.716
8	0.120	0.272	0.457	0.188	0.425	0.714
9	0.120	0.273	0.458	0.188	0.427	0.716
10	0.120	0.273	0.458	0.188	0.427	0.716
			mean	0.188	0.426	0.715
			mean - blank*	0.121	0.359	0.648
			conc M	1.407E-05	4.156E-05	7.509E-05
			DF (if required)	1.407E-05	4.156E-05	7.509E-05
			µM	14.069	41.560	75.093
			µM /Hr (µM /3)	4.690	13.853	25.031
				replicate mean		14.525
				replicate standard deviation		10.187

All raw data can be found at https://doi.org/10.7488/ds/7499

Table S1. Literature data for the comparison of proteinases for keratinase activity with reference to Proteinase K as seen in **Figure1.**

Name	Relative Activity (%)	Std Dev	Calculated Relative Activity (%)	PK Value	Reported Figure	Units	Substrate	Reference
Bacillus sp. MTS	**1740**		1740.00	0.5	8.7	U/mg	Wool	1
NAPase*	**285**		284.62	0.26	0.74	Abs at 280 nm	Keratin	2
AH-101 protease*	**250**		250.00	0.2	0.5	Abs at 660 nm	Keratin	3
Ker A	**192**		192.31	52	100	%	Azokeratin (feather)	4
SFP2	**174**		174.12	34	59.2	U/mg min	Synthetic peptide	5
*Streptomyces pactum DSM 40530**	**150**		150.00	0.8	1.2	Abs at 620 nm	Keratin azure	6
SAKase	**119**		118.72	167.2	198.5	U/mg	Keratin azure	7
Proteinase K	**100**		-		-	-	-	1-9
SGPBc	**83**		82.66	167.2	138.2	U/mg	Keratin azure	7
Paecilomyces marquandii	**71**		70.66	35.1	24.8	U/mg/min	Skin keratin	8
Elastase	**57**	**47**	23.36	35.1	8.2	U/mg/min	Skin keratin	8
			90.38	52	47	%	Azokeratin (feather)	4
Subtilisin Carlsberg*	**42**	**49**	76.92	0.26	0.2	Abs at 280 nm	Keratin	2
			7.62	722	55	Keratinolytic activity (KU/mg)	keratin azure	9
Doratomyces microsporus	**42**		41.60	35.1	14.6	U/mg/min	Skin keratin	8
Trypsin	**25**	**21**	4.12	34	1.4	U/mg min	Synthetic peptide	5
			11.97	35.1	4.2	U/mg/min	Skin keratin	8
			30.74	722	222	Keratinolytic activity (KU/mg)	keratin azure	9
			51.92	52	27	%	Azokeratin (feather)	4
Subtilisin	**19**		18.80	35.1	6.6	U/mg/min	Skin keratin	8
Papin	**13**		13.46	52	7	%	Azokeratin (feather)	4
Protease D-1	**11**		11.50	722	83	Keratinolytic activity (KU/mg)	keratin azure	9
Chymotrypsin	**10**	**6**	5.00	34	1.7	U/mg min	Synthetic peptide	5
			8.55	35.1	3	U/mg/min	Skin keratin	8
			17.17	167.2	28.7	U/mg	Keratin azure	7

Table S2. Definitions of units of keratinase activity used in Keratin Azure assays in recent publications, showing the variability between how the units are defined, and the conditions under which they are measured. All studies optimise buffers, temperatures, and pH for the enzyme.

Increase in absorbance at 595 nm	Over time period	Δ Abs_{595} hr^{-1}	Enzyme concentration	Keratin Azure / mg mL^{-1}	Use of TCA to quench	Ref.
0.001	90 min	0.00067	Not specified	4	Yes	10
0.001	1 hour	0.001	Not specified	20	Yes	11
0.01	1 hour	0.01	Not specified	4	No	12
0.01	1 hour	0.01	Not specified	4	Yes	13
0.01	1 hour	0.01	Unit defined per mL enzyme, concentration not specified	4	No	14
0.01	1 hour	0.01	15 µg mL^{-1}	4	No	15
0.01	1 hour	0.01	Not specified	5	No	16-18
0.01	1 hour	0.01	Not specified	5	Yes	19
0.01	30 min	0.02	1 mg ml^{-1}	10	No	20
0.01	20 min	0.03	Not specified	5	Yes	21
0.01	15 min	0.04	Not specified	5	Yes	22,23
0.1	1 hour	0.1	Not specified	0·4% (w/v)	No	24
0.1	1 hour	0.1	Not specified	5	No	25
0.01	1 min	0.6	Not specified	5	No	26-28
0.1	1 min	6	Not specified	5	No	29-32

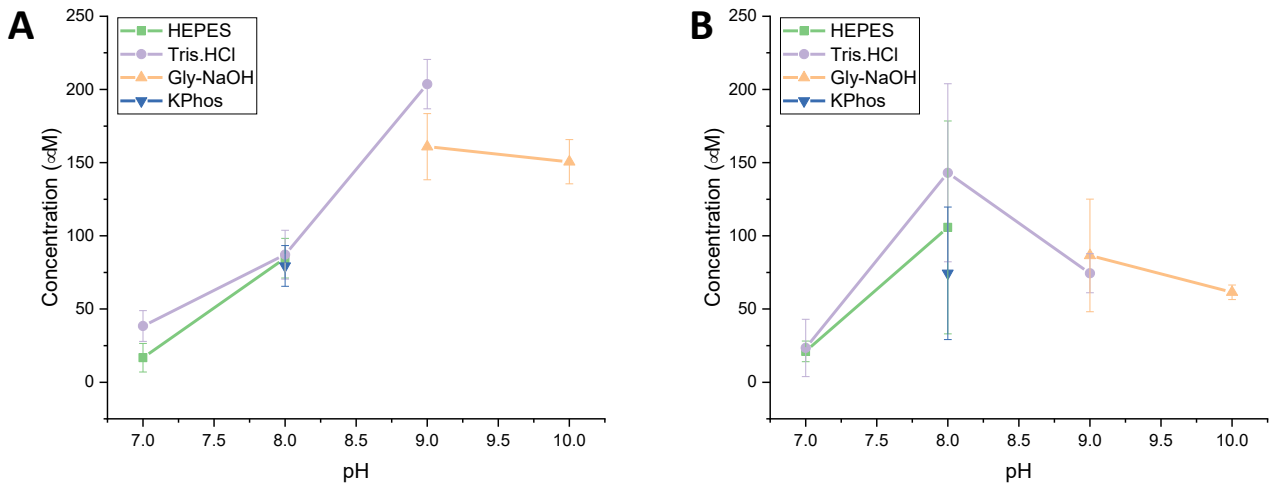

Figure S1. Proteinase K (PK) and Subtilisin Carlsberg (SC) buffer and pH screen. The reaction: A PK or B SC (50 μM) added to buffer (50 mM, HEPES/Tris.HCl/Gly-NaOH or KPhos) at pH ranges 7.0 – 10.0 containing 0.5 % w/v KA (5.0 mg), the reaction was then shaken for 3 hrs at 50 °C on a thermoshaker.

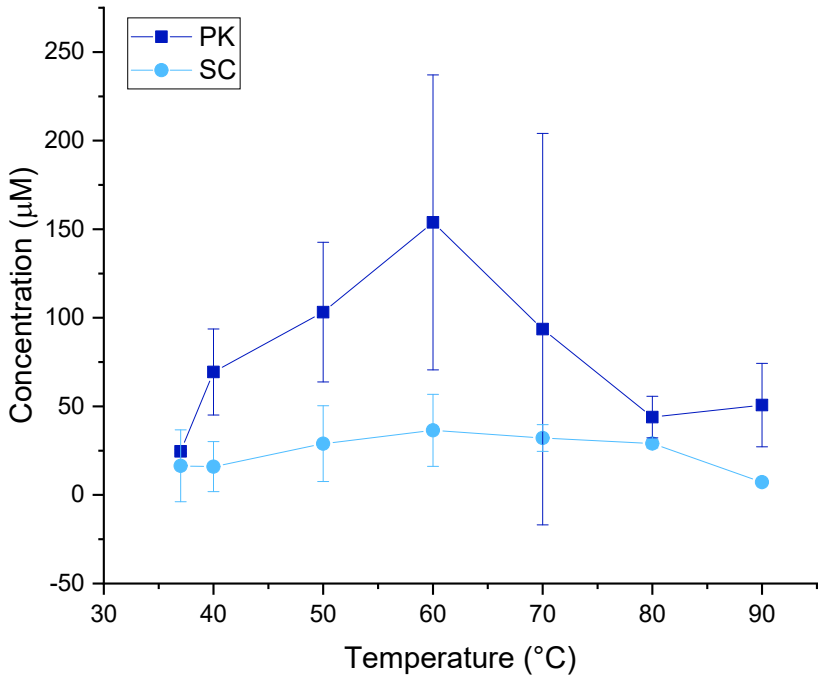

Figure S2. Proteinase K and Subtilisin Carlsberg temperature screen. The reaction: PK or SC (50 μM) added to buffer (50 mM, Tris.HCl pH 8.0 (SC) or 9.0 (PK)) containing 0.5 % w/v KA (5.0 mg), the reaction was then shaken for 3 hrs at temperature ranging 37-90 °C on a thermoshaker.

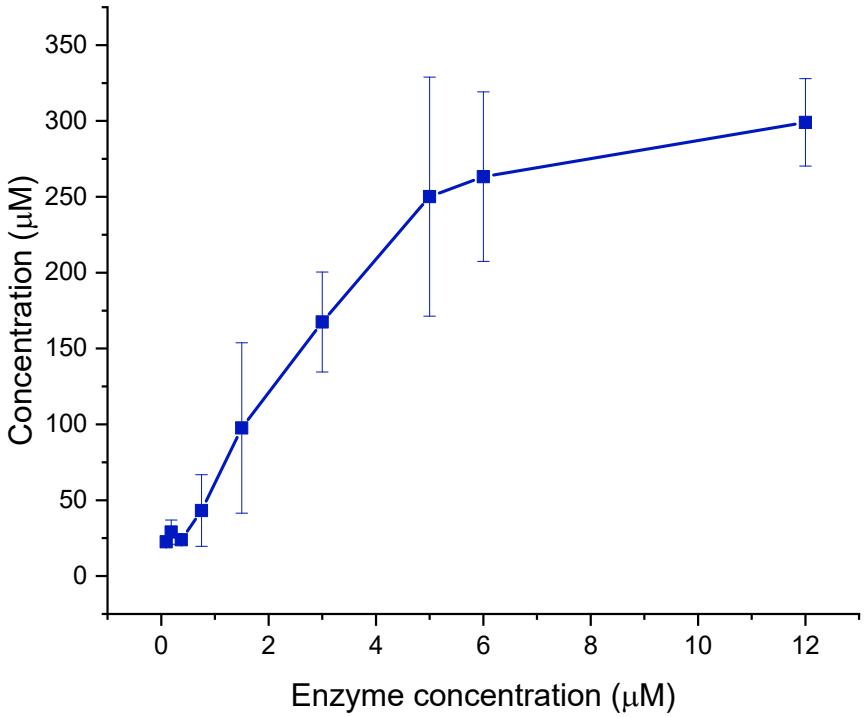

Figure S3. Proteinase K enzyme concentration screen. The reaction: PK (0-12 μM) added to buffer (50 mM, Tris.HCl pH 9.0) containing 0.5 % w/v KA (5.0 mg), the reaction was then shaken for 3 hrs at 60 °C on a thermoshaker.

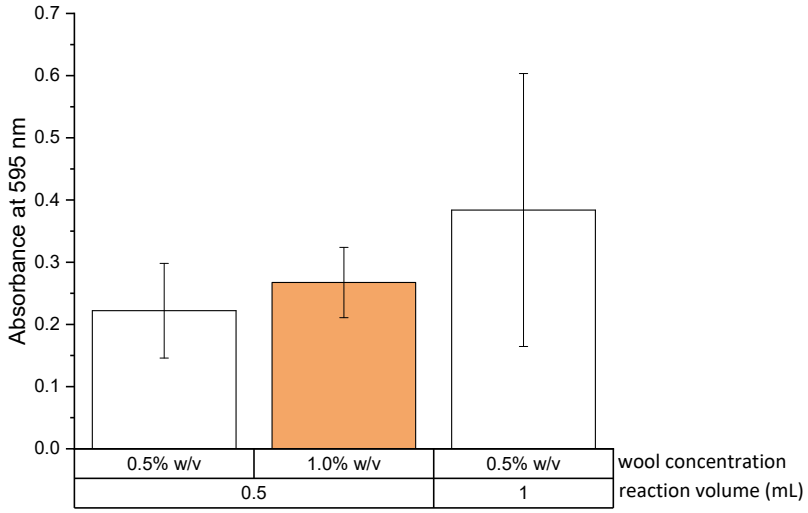

Figure S4. Investigation of reaction volume and KA concentration. The KA was prepared by adding PK (50 μM) to buffer (Tris-HCl buffer, 50 mM, pH 8.0) containing 0.5 or 1 % w/v KA to a total reaction volume of 0.5 or 1 mL, the reaction Eppendorfs were shaken for 3 hrs at 50 °C on a thermoshaker. Samples were filtered (17 mm 0.45 μm PTFE filter). 200 μL was transferred to a 96-well plate and were measured using a plate reader at 595 nm. Created in Origin.

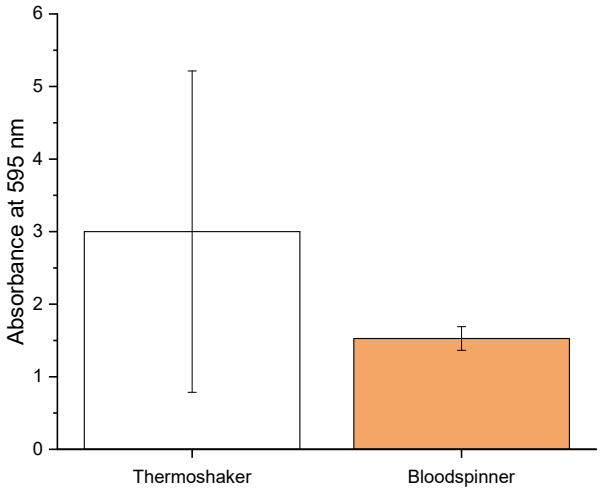

Figure S5. Investigation of mixing technique. The KA was prepared by adding PK (50 μM) to buffer (Tris-HCl buffer, 50 mM, pH 8.0) containing 1 % w/v KA to a total reaction volume of 0.5 mL, the reaction Eppendorfs were shaken for 3 hrs at 50 °C on a thermoshaker or bloodspinner. Samples were filtered (17 mm 0.45 μm PTFE filter). 200 μL was transferred to a 96-well plate and were measured using a plate reader at 595 nm. Created in Origin.

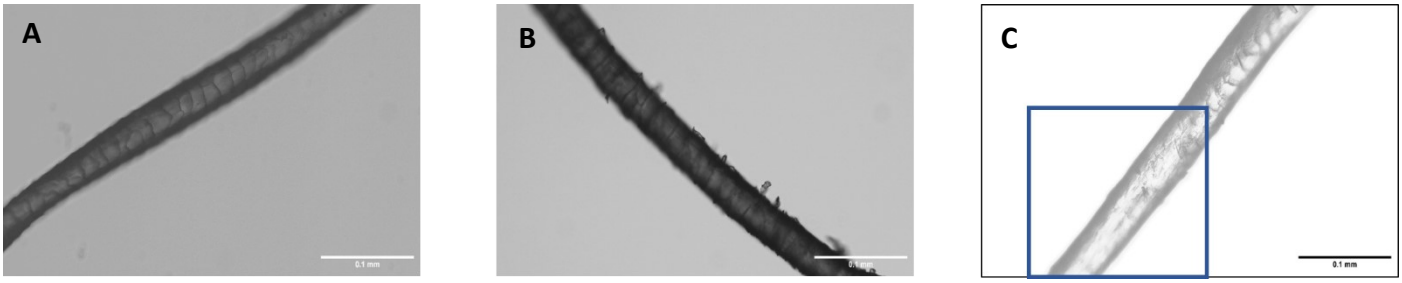

Figure S6. Effect of PK on KA. A. Starting KA. B&C. Post-incubation fibre taken at 20x magnification. Created in ImageJ.

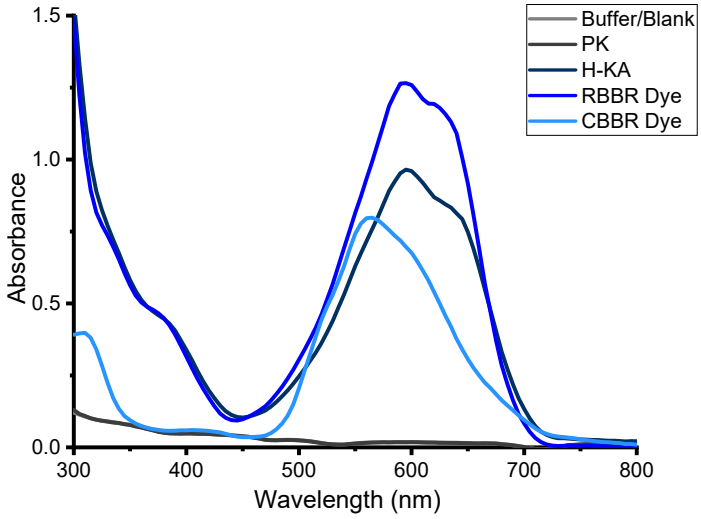

Figure S7. UV-Vis spectra comparing the absorbance of PK, hydrolysed KA, RBBR dye (150 µM) and coomassie brilliant blue R (CBBR) dye showing the differences between RBBR and CBBR dye (25 µM). The hydrolysed KA was prepared by adding PK (50 µM) to buffer (Tris-HCl buffer, 50 mM, pH 8.0) containing 0.5% w/v KA, the and the reaction Eppendorfs shaken for 3 hrs at 50 °C on a thermoshaker. Created in Origin.

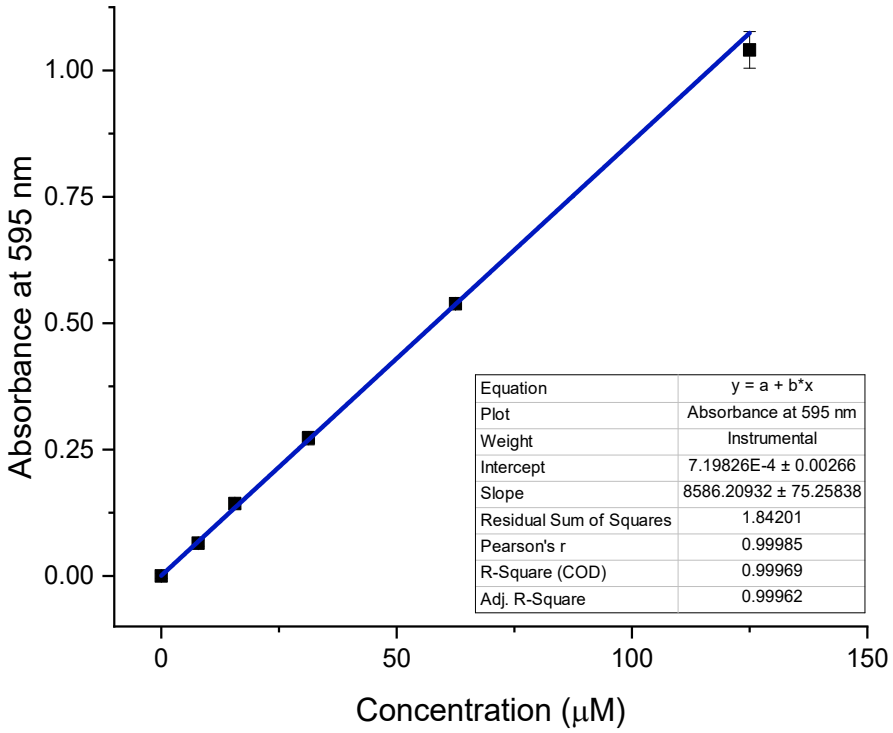

Figure S8. Calibration curve of RBBR Dye in 50 mM Tris-HCl, pH 8.0 buffer. Making a stock of 1 mM RBBR Dye in 50 mM Tris-HCl, pH 8.0 buffer. A serial dilution was performed to get concentrations between 7.8 -125.0 μM. 200 μL was transferred to a 96-well plate and were measured using a plate reader at 595 nm. A triplicate blank reading was also taken, averaged and subtracted from each point. Created in Origin.

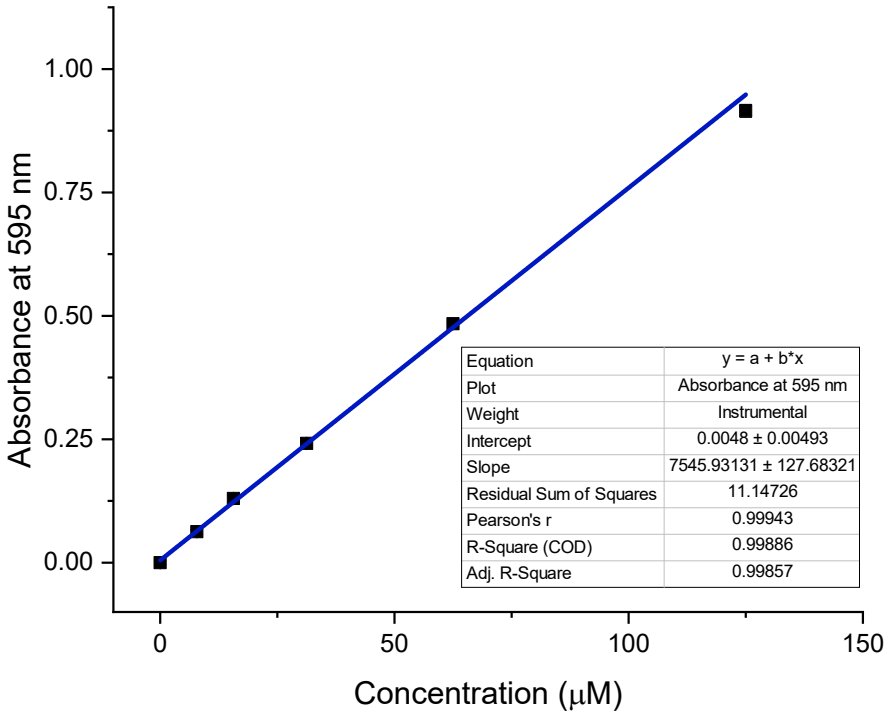

Figure S9. Calibration curve of RBBR Dye in 50 mM Tris-HCl, pH 9.0 buffer. Making a stock of 1 mM RBBR Dye in 50 mM Tris-HCl, pH 9.0 buffer. A serial dilution was performed to get concentrations between 7.8 -125.0 μM. 200 μL was transferred to a 96-well plate and were measured using a plate reader at 595 nm. A triplicate blank reading was also taken, averaged and subtracted from each point. Created in Origin.

Calculation using Beer Lambert law

$A = \varepsilon lc$

Where

A = absorbance at the measured wavelength, which is unitless

ε = the molar absorption coefficient of the species at the measured wavelength, in M^{-1} cm^{-1}

l = the path length of the cuvette, in cm

c = the concentration of the compound being measured, in M (mol L^{-1})

To convert the results of the Keratin Azure assay from absorbance to the concentration of dye released, rearrange the equation and substitute the experimental values in;

$$c = \frac{0.078}{8586\ M^{-1}\ cm^{-1} \times 0.64\ cm} = 1.419 \times 10^{-5} M$$

Calculation Limit of Detection (LOD)

$x - x_B = 3s_B$

Where:

x = sample signal

x_B = analytical blank signal

s_B = standard deviation of the analytical blank

Concentration (μM)	x	x_B	s_B	$3s_B$	LOD reached
2.0 (pH 8.0)	0.014	0.054	0.005	0.015	No
3.9 (pH 8.0)	**0.026**	**0.054**	**0.005**	**0.015**	**Yes**
2.0 (pH 9.0)	0.002	0.058	0.004	0.012	No
3.9 (pH 9.0)	**0.023**	**0.058**	**0.004**	**0.012**	**Yes**

Calculation Limit of Quantification (LOQ)

$x - x_B = 10s_B$

Concentration (μM)	x	x_B	s_B	$10s_B$	LOQ reached
3.9 (pH 8.0)	0.026	0.054	0.005	0.051	No
7.8 (pH 8.0)	**0.065**	**0.054**	**0.005**	**0.051**	**Yes**
3.9 (pH 9.0)	0.023	0.058	0.004	0.042	No
7.8 (pH 9.0)	**0.062**	**0.058**	**0.004**	**0.042**	**Yes**

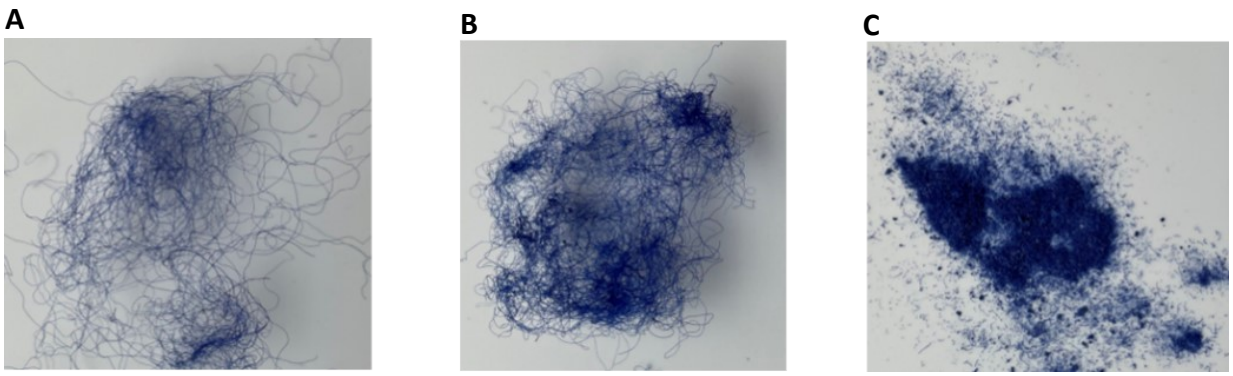

Figure S10. Pre-treated KA. A. Unprocessed (UP). B. Autoclaved KA (AC, 130 °C for 15 min). C. milled KA (M, 30 Hz/s for 3 min).

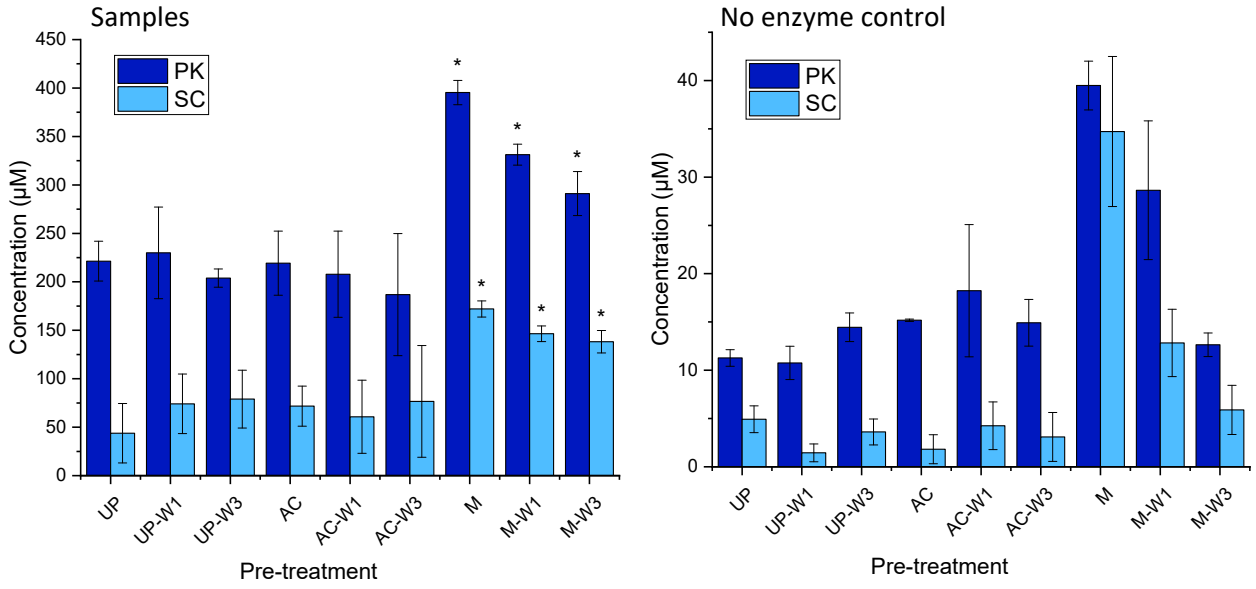

Figure S11. Endpoint concentration of dye released during the keratin azure assay with various pre-treated substrates and the no enzyme control. Unprocessed (UP), Autoclaved KA (AC, 130 °C for 15 min) and milled KA (M, 30 Hz/s for 3 min), with and without being washed with distilled water (1 mL) once (UP-W1, AC-W1, M-W1), or three times (UP-W3, AC-W3,M-W3). The wash was discarded. To 1.0% w/v of treated KA: buffer (Tris-HCl buffer, 50 mM, pH 9.0/8.0) and PK/SC (50 µM) were added. After being mixed in an Eppendorf for3 hrs at 60 °C in an incubator, samples were filtered (17 mm 0.45 µm PTFE filter). 200 µL was transferred to a 96-well plate and was measured using a plate reader at 595 nm. When compared to UP, $p<0.05$*. Created in Origin.

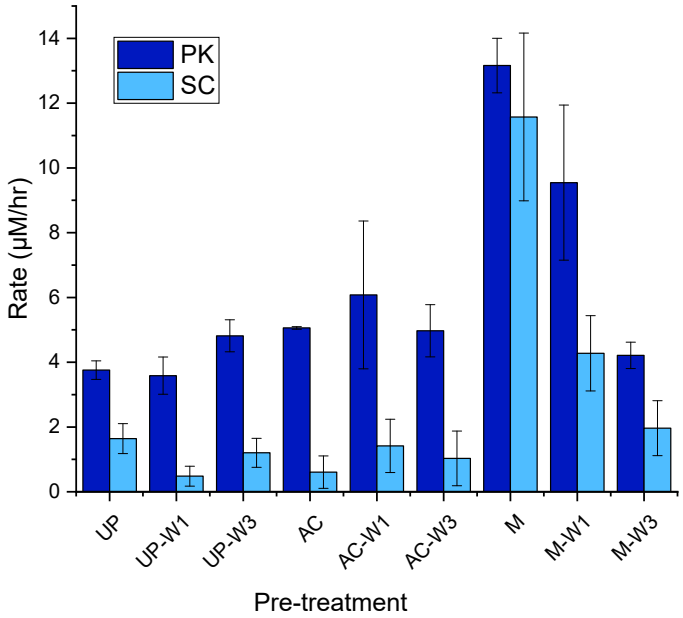

Figure S12. Rate of dye released during the no enzyme keratin azure assay control with various pre-treated substrates. Unprocessed (UP), Autoclaved KA (AC, 130 °C for 15 min) and milled KA (M, 30 Hz/s for 3 min), with and without being washed with distilled water (1 mL) once (UP-W1, AC-W1, M-W1), or three times (UP-W3, AC-W3,M-W3). The wash was discarded. To 1.0% w/v of treated KA: buffer (Tris-HCl buffer, 50 mM, pH 9.0/8.0) was added. After being mixed in an Eppendorf for3 hrs at 60 °C in an incubator, samples were filtered (17 mm 0.45 µm PTFE filter). 200 µL was transferred to a 96-well plate and was measured using a plate reader at 595 nm. Created in Origin.

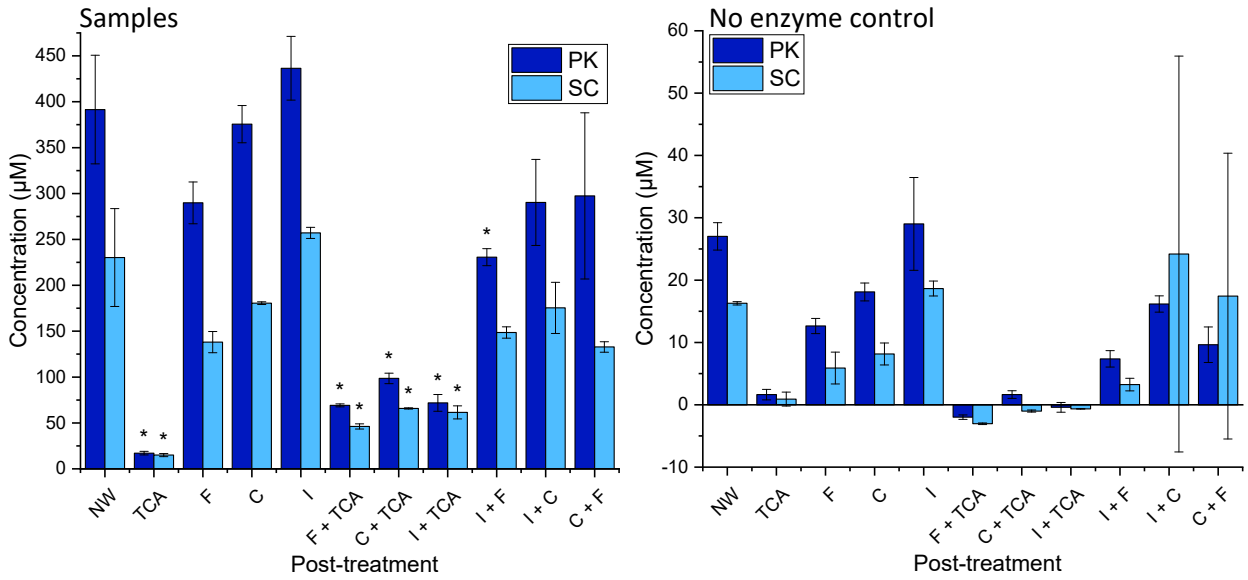

Figure S13. Endpoint concentration of dye released in keratin azure assay post-treatment using 11 different techniques and the no enzyme control. Milled KA (30 Hz/s for 3 min) was washed with distilled water (1 mL) three times. The wash was discarded. To the KA (1.0% w/v) buffer (Tris-HCl buffer, 50 mM, pH 9.0/8.0) and PK/SC (50 µM) were added. After being mixed in an Eppendorf for3 hrs at 60 °C in an incubator, samples were iced (30 mins), centrifuged (13 kRPM, 10 mins), filtered (17 mm 0.45 µm PTFE filter) or treated with TCA (10% w/v, 3:1 TCA:reaction sample, 30 min incubation the centrifuge 10 min, 13 kRPM). 200 µL was transferred to a 96-well plate and was measured using a plate reader at 595 nm. $p<0.05$*. Created in Origin.

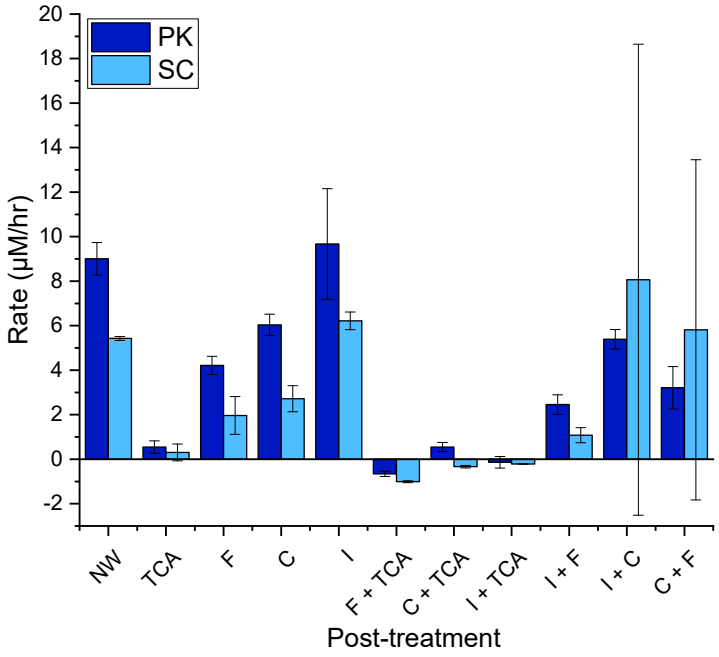

Figure S14. Rate of dye released during the no enzyme keratin azure assay control post-treatment using 11 different techniques Milled KA (30 Hz/s for 3 min) washed with distilled water (1 mL) three times. The wash was discarded. To the KA (1.0% w/v) buffer (Tris-HCl buffer, 50 mM, pH 9.0/8.0) was added. After being mixed in an Eppendorf for3 hrs at 60 °C in an incubator, samples were iced (30 mins), centrifuged (13 kRPM, 10 mins), filtered (17 mm 0.45 µm PTFE filter) or treated with TCA (10% w/v, 3:1 TCA:reaction sample, 30 min incubation the centrifuge 10 min, 13 kRPM). 200 µL was transferred to a 96-well plate and was measured using a plate reader at 595 nm. Created in Origin.

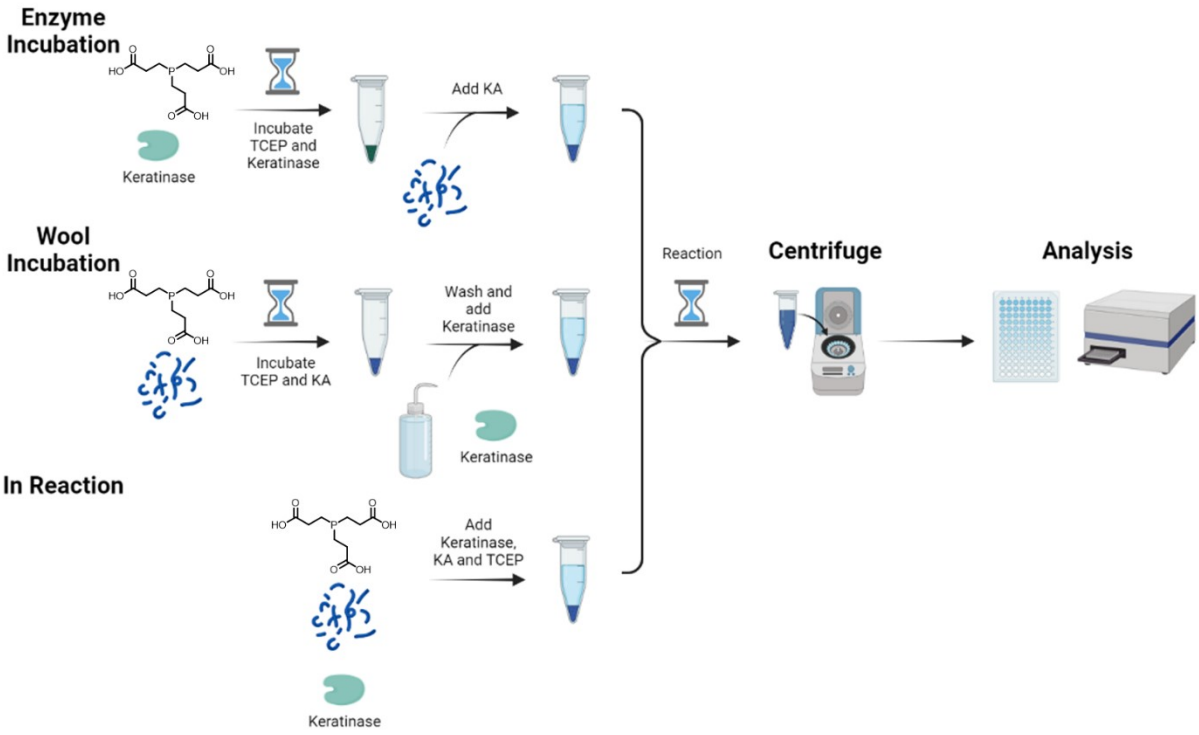

Figure S15. TCEP experimental design. Three different reaction procedures testing the effect of TCEP on the KA assay. Created with BioRender.com.

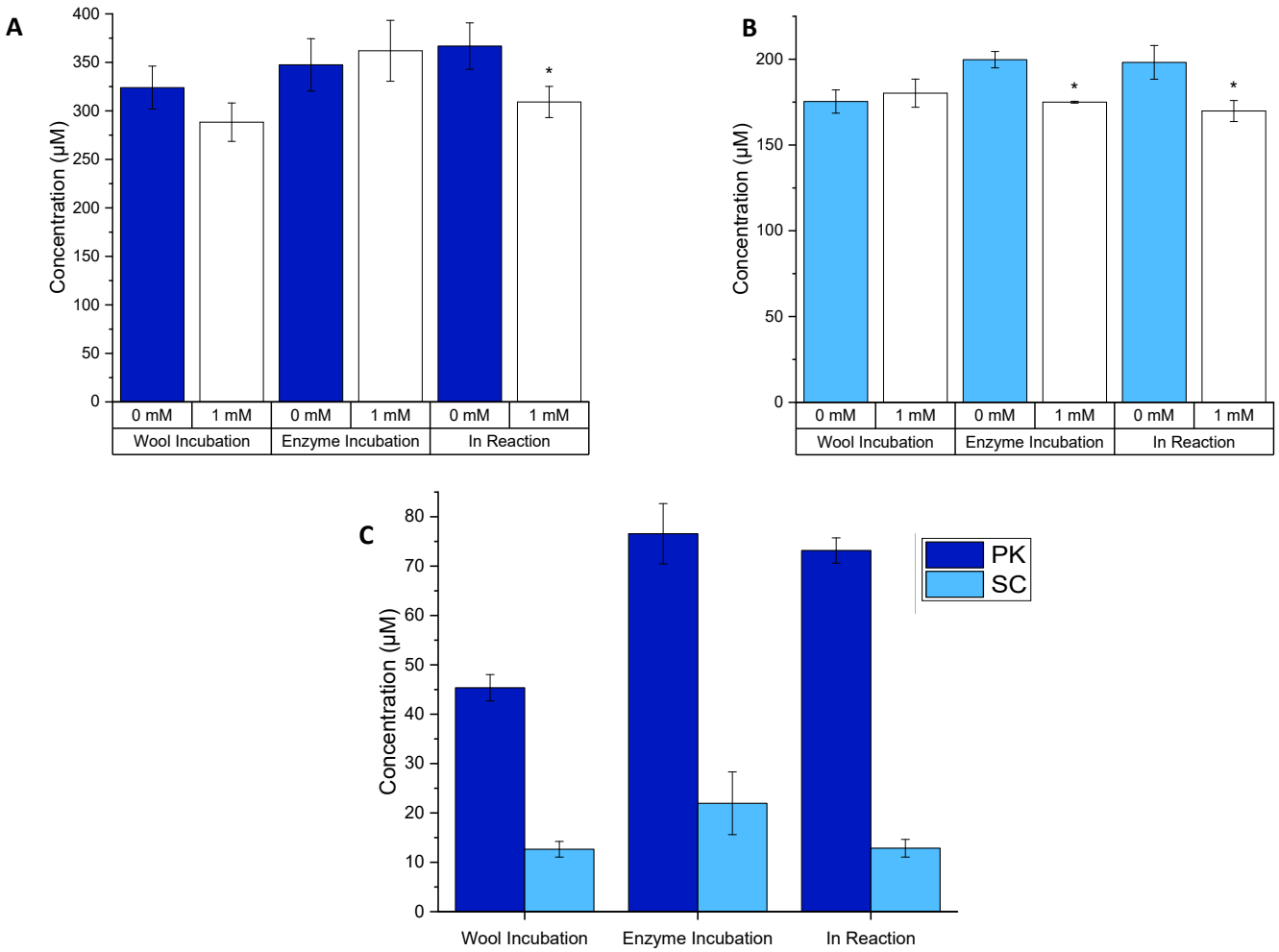

Figure S16. The endpoint effects of TCEP on KA and keratinase or both with keratinase A: PK, B: SC and C: no enzyme control. Milled KA (30 Hz/s for 3 min) washed with distilled water (1 mL) three times. The wash was discarded. TCEP was added to wool only (TCEP:50 μL, 10 mM), enzyme only (TCEP: 50 μL, 10 mM, enzyme 50 μL, 50 μM, incubated for 30 min at 25 °C with 400 μL of buffer) or in the reaction (TCEP:50 μL, 10 mM) as illustrated in **Figure S8**. In the reactions were also KA (1.0% w/v) buffer (Tris-HCl buffer, 50 mM, pH 9.0/8.0) and PK/SC (50 μM). After being mixed in an Eppendorf for 3 hrs at 60 °C in an incubator, samples were centrifuged (13 kRPM, 10 mins). 200 μL was transferred to a 96-well plate and was measured using a plate reader at 595 nm. $p<0.05$*. Created in Origin.

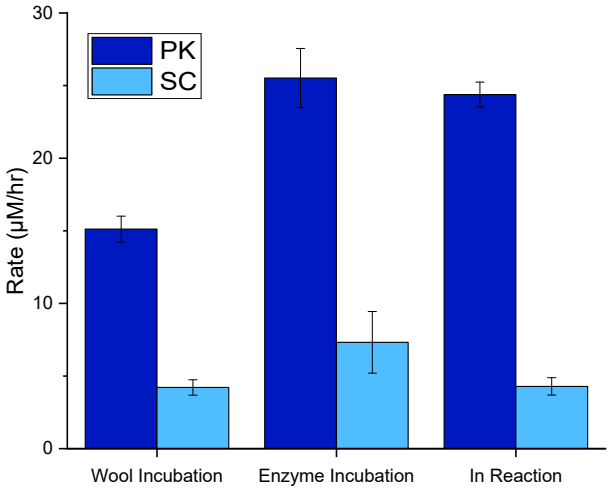

Figure S17. The effects of TCEP on the rate of keratinase activity with different incubations, no enzyme control. Milled KA (30 Hz/s for 3 min) washed with distilled water (1 mL) three times. The wash was discarded. TCEP was added to wool only (TCEP:50 μL, 10 mM), enzyme only (TCEP: 50 μL, 10 mM, enzyme 50 μL, 50 μM, incubated for 30 min at 25 °C with 400 μL of buffer) or in the reaction (TCEP:50 μL, 10 mM) as illustrated in **Figure S8**. In the reactions were also KA (1.0% w/v) buffer (Tris-HCl buffer, 50 mM, pH 9.0/8.0) and PK/SC (50 μM). After being mixed in an Eppendorf for 3 hrs at 60 °C in an incubator, samples were centrifuged (13 kRPM, 10 mins). 200 μL was transferred to a 96-well plate and was measured using a plate reader at 595 nm. $p<0.05$*. Created in Origin.

References

1. S. Rahayu, D. syah and M. T. Suhartono, Biocatal. Agric. Biotechnol., 2012, 1, 152-158.
2. S. Mitsuiki, M. Ichikawa, T. Oka; M. Sakai, Y. Moriyama, Y Sameshima, M. Goto and K. Furukawa, Enzyme Microb. 2004, 34, 482-489.
3. H. Takami, Akiba, T.; Horikoshi, K. Appl. Microbiol. Biotechnol., 1990, 33, 519-523.
4. X. Lin, C.G. Lee, E.S. Casale, J. C. H. Shih, Appl Environ Microbiol., 1992, 58, 3271-3275.
5. J. Li, P.J. Shi, X.Y. Han, K. Meng, P.L. Yang, Y.R. Wang, H.Y. Luo, N.F. Wu, B. Yao and Y.L. Fan, Protein Expr. Purif., 2007, 54, 79-86.
6. B. Böckle, B. Galunsky, and R. Müller, Appl Environ Microbiol., 1995, 61, 3705-3710.
7. P. Bressollier, F. Letourneau, M. Urdaci and B. Verneuil, Appl Environ Microbiol.,1999, 65, 2570-2576.
8. H. Gradišar, J. Friedrich, I. Križaj and R. Jerala, Appl Environ Microbiol., 2005, 71, 3420-3426.
9. S. Yamamura, Y. Morita, Q. Hasan, K. Yokoyama and E. Tamiya, BBRC, 2002, 294, 1138-1143.
10. Y. Jagadeesan, S. Meenakshisundaram, V. Saravanan and A. Balaiah, Int. J. Biol. Macromol., 2020, 163, 135-146.
11. L.X. Lv, M.H. Sim, Y.D. Li, J. Min, W.H. Feng, W.J. Guan and Y.Q. Li, Process Biochem., 2010, 45, 1236-1244.
12. A. B. Vermelho, QA.M. Mazotto, S.M.L. Cedrola, in Methods to Determine Enzymatic Activity, ed. A. B. Vermelho and D. Couri, Bentham Science Publishers, UAE, 1st edn, 2013, ch. 10, pp. 226-261.
13. A. Gegeckas, R. Gudiukaitė, J. Debski and D. Citavicius, Int. J. Biol. Macromol., 2015, 75, 158-165.
14. J.M. Kuo, J.I. Yang, W.M. Chen, M.H. Pan, M.L. Tsai, Y.J. Lai, A. Hwang, B. S. Pan and C.Y. Lin, Int. Biodeterior, 2012, 70, 111-116.
15. X. Liang, Y. Bian, X.F. Tang, G. Xiao and B. Tang, Appl. Microbiol. Biotechnol., 2010, 87, 999-1006.
16. N. E. Nnolim, L. Mpaka, A. I. Okoh and U. U. Nwodo, Microorganisms, 2020, 8, 1304.
17. X. Chen, B. Zhou, M. Xu, Z. Huang, G. Jia and H. Zhao, Biologia, 2015, 70, 157-164.
18. N. E. Nnolim, N. Ntozonke, A. I. Okoh and U. U. Nwodo, Biochimie, 2020, 177, 53-62.
19. F. Akram, I.U. Haq and Z. Jabbar, Int. J. Biol. Macromol., 2020, 164, 371-383.
20. T. Sone, Y. Haraguchi, A. Kuwahara, T. Ose, M. Takano, A. Abe, M. Tanaka, I. Tanaka and K. Asano, Protein Pept. Lett., 2015, 22, 63-72.
21. S. E. Tork, Y. E. Shahein, A. E. El-Hakim, A. M. Abdel-Aty and M. M. Aly, Int. J. Biol. Macromol., 2016, 86, 189-196.
22. V. A. Pawar, A. S. Prajapati, R. C. Akhani, D. H. Patel and R. B. Subramanian, 3 Biotech, 2018, 8, 107.
23. S. E. Tork, Y. E. Shahein, A. E. El-Hakim, A. M. Abdel-Aty and M. M. Aly, Int. J. Biol. Macromol., 2013, 55, 169-175.
24. F. Letourneau, V. Soussotte, P. Bressollier, P. Branland and B. Verneuil, Lett Appl Microbiol, 1998, 26, 77-80.
25. N. E. Nnolim, A.I. Okoh and U.U. Nwodo, Environ. Technol. Innov., 2021, 21, 101285.
26. K. Bouacem, A. Bouanane-Darenfed, N. Zaraî Jaouadi, M. Joseph, H. Hacene, B. Ollivier, M.-L. Fardeau, S. Bejar and B. Jaouadi, Int. J. Biol. Macromol., 2016, 86, 321-328.
27. M.B. Elhoul, N.Z. Jaouadi, H. Rekik, M.O. Benmrad, S. Mechri, E. Moujehed, S. Kourdali, M. El Hattab, A. Badis, S. Bejar and B. Jaouadi, Int. J. Biol. Macromol., 2016, 92, 299-315.
28. N. Zaraî Jaouadi, H. Rekik, M.B. Elhoul, F.Z. Rahem, C.G. Hila, H.S.B. Aicha, A. Badis, A. Toumi, S. Bejar and B. Jaouadi, Int. J. Biol. Macromol., 2015, 79, 952-964.
29. S. Hamiche, S. Mechri, L. Khelouia, R. Annane, M. El Hattab, A. Badis and B. Jaouadi, Int. J. Biol. Macromol., 2019, 122, 758-769.
30. A. Habbeche, B. Saoudi, B. Jaouadi, S. Haberra, B. Kerouaz, M. Boudelaa, A. Badis and A. Ladjama, J. Biosci. Bioeng., 2014, 117, 413-421.
31. N. Z. Jaouadi, H. Rekik, A. Badis, S. Trabelsi, M. Belhoul, A. B. Yahiaoui, H. B. Aicha, A. Toumi, S. Bejar and B. Jaouadi, PLOS ONE, 2013, 8, e76722.
32. A. Benkiar, Z. J. Nadia, A. Badis, F. Rebzani, B. T. Soraya, H. Rekik, B. Naili, F. Z. Ferradji, S. Bejar and B. Jaouadi, Int. Biodeterior. Biodegrad., 2013, 83, 129-138.
33. B. Jaouadi, B. Abdelmalek, D. Fodil, F. Z. Ferradji, H. Rekik, N. Zaraî and S. Bejar, Bioresour. Technol., 2010, 101, 8361-8369.