

1 **Optimisation of fungal cellulase production from textile waste using** 2 **experimental design**

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10

11 **Abstract**

12 Abundant textile waste raises increasing concerns worldwide in developing novel
13 circular textiles approach. This study investigated the optimum cellulase production
14 from textile waste by *Aspergillus niger* CKB. Textile wastes consisting of cotton and
15 polyester in various ratios were used as low-cost feedstocks. Three types of
16 cultivation media were compared in solid state fermentation, in which Mandels
17 medium with yeast extract was selected due to their superior cellulase production.
18 Conditions including moisture, pH, inoculum size and organic nitrogen were
19 evaluated and optimised via Response Surface Methodology. Supplementary carbon
20 sources and cellulase inducers were also employed to enhance the fungal growth and
21 cellulase production. The results indicated that the optimised fermentation method
22 significantly improved cellulase production efficiency and enzyme activity by 88.7%
23 and 25.8%, respectively. The maximum cellulase activity reached 1.56 FPU g⁻¹ in 6
24 days. The outcomes of this study led to efficient recovery of glucose and polyester,
25 which could contribute to a closed-loop recycling strategy for the textile industry and
26 enable the transition towards an advanced circular textiles economy.

27 **Keywords:** Cotton; Fungal cellulase; Response surface methodology; Solid state
28 fermentation; Textile waste

29

30 **1. Introduction**

31 Globally increasing consumption of textiles and fashion products led to a huge
32 accumulation of textile waste and serious environmental problems (Caulfield, 2009).

33 In 2015-2016, the annually worldwide production volume of textile fibres reached 80-
34 90 million tonnes and it is forecasted to exceed 100 million tonnes soon (Lenzing
35 Corporation, 2016). The garment, textile and fashion industries are greatly pollutive
36 and it is evaluated as the second most polluting industry in the world, following the
37 petroleum industry (Sweeny, 2015). On the global average, 32 kg of textile wastes are
38 discarded per capita each year, of which the majority (around 85%) is directly
39 disposed by landfill or incineration, leading to soil contaminations and greenhouse
40 gases emission (Textile Exchange, 2012). Waste & Resource Action Programme
41 (WRAP, UK) evaluated that around 95% of landfilled textile waste is recyclable,
42 whereas only 14-15% recycling rate has been reached (WRAP, 2016). Nowadays,
43 textile recycling mainly relies on second-hand dumping and downcycling into rags,
44 which actually do not capture values from textiles. In order to develop a new textiles
45 economy based on a circular system, global textile manufacturers such as H&M are
46 developing efficient recycling strategies to capture the embodied value of fibres
47 (H&M, 2017).

48 In view of textile materials, cotton and polyester (PET) are the most widely used
49 types of fibres. Approximately 35-40% of textile waste is comprised of cotton, which
50 is a potential cellulosic feedstock for bioproducts, such as enzymes, ethanol and

51 biogas (Jeihanipour *et al.*, 2010; Shen *et al.*, 2013). Pensupa *et al.* (2017) summarised
52 the development of utilising textile waste through processes involving pretreatment,
53 saccharification and fermentation. Lignocellulosic wastes (*e.g.* agricultural waste and
54 horticultural waste) have been developed as low-cost substrates for cellulase
55 production in the last decade (Bansal *et al.*, 2012; Xin and Geng, 2010). The
56 feasibility of using cotton-based textile material in this area was investigated for the
57 first time in our recent study (Hu *et al.*, 2017), which produced cellulase from textile
58 waste (cotton and PET blends) by solid state fermentation (SSF).

59 Fermentation conditions of SSF are crucial for microbial growth and metabolic
60 activity. There is a direct relationship between substrate, fermentation conditions and
61 cellulase production (Yoon *et al.*, 2014). As textile waste is a newly applied substrate
62 in SSF, the suitable conditions deserve a comprehensive optimisation to maximise
63 cellulase production. The affecting parameters in SSF include fermentation medium,
64 temperature, moisture content, pH and supplementary nutrients (Sukumaran *et al.*,
65 2005). Fermentation medium has been stressed in literature as it has profound effects
66 on types and concentrations of fungal cellulase produced (Yoon *et al.*, 2014).
67 Moisture content of the medium is essential for fungal growth and metabolism in SSF,
68 as well as affects the diffusion of nutrients and air. Low moisture content limits the
69 solubility of nutrients while high moisture level could decrease the porosity of
70 substrate and oxygen transfer (Kumar *et al.*, 2011). Besides, as moisture requirement
71 is directly related to the physical characters of substrate such as surface structure and
72 water holding capacity, it is necessary to consider the nature of substrate applied in
73 SSF when optimising the moisture condition (Orzua *et al.*, 2009). Inoculum size is
74 another crucial factor in enzyme production. At a low inoculum size, limited conidial
75 cells cannot fully utilise the nutrients in medium, leading to poor cellulase

76 biosynthesis (Bansal *et al.*, 2012). In contrast, excessive inoculum size usually causes
77 nutritional imbalance and an anaerobic environment under tremendous fungal growth
78 (Bansal *et al.*, 2012). Other factors such as pH and temperature could also influence
79 enzyme production and activity in SSF. The interactions between different variables
80 should also be taken into account. For instance, the combination of moisture content
81 and inoculum size plays a vital part in affecting the dynamics of microbial growth on
82 solid substrates (*i.e.* colonisation of fungus, assembly of solid substrates), as well as
83 mass and heat transfer in SSF (Ustok *et al.*, 2007). In addition, supplementary carbon
84 sources have been suggested to support fungal growth and to promote cellulase
85 activity (Liang *et al.*, 2012; Olsson *et al.*, 2003). As an inducible enzyme, cellulase
86 production can be initiated or enhanced by appropriate inducers under specific
87 conditions, which allows a more controlled gene expression of the enzyme
88 (Sukumaran *et al.*, 2005).

89 Response Surface Methodology (RSM) is one of the most practical methods used in
90 system optimisation (Kumar *et al.*, 2011). Through experimental design and
91 modelling, RSM not only identifies the effect of individual variables, but also
92 evaluates the interaction of various parameters to seek the optimal solution. This
93 method has been widely applied in optimisation studies of biotechnology and
94 industrial processes (Kumar *et al.*, 2011; Soleimaninanadegani *et al.*, 2014).
95 Levin *et al.* (2008) enhanced lignocellulosic enzyme production by optimisation of
96 fermentation conditions through RSM, endo-xylanase activity was enhanced by
97 50.8%. Yasmeen *et al.* (2013) investigated lignocellulosic enzyme production using
98 agricultural wastes with the cultivation conditions optimised by a five-factor-five-
99 level Central Composition Design (CCD) model in RSM. The activities of lignin

100 peroxisase, manganese peroxidase and laccase were improved by 26.1%, 16.4% and
101 34.2%, respectively.

102 In order to develop a novel recycling method of textile waste, a recent research
103 project funded by the Innovation Technology Commission in Hong Kong entitled
104 “Textile Waste Recycling by Biological Method” is currently conducted by our group
105 for recovery of cellulose and PET from blended materials. Based on our preliminary
106 research of cellulase production from textile waste (Hu *et al.*, 2017), SSF conditions
107 were systematically optimised via experimental design in this study. Different
108 cultivation media and physical conditions were compared in terms of cellulase
109 activity. Effect of supplementary carbon sources and presence of inducers on cellulase
110 production were also investigated. Finally, an overall material balance of the
111 bioconversion process was evaluated.

112

113 **2. Materials and methods**

114 2.1 Textile waste and microorganism

115 Three different types of cotton/PET blended textile wastes provided by H&M
116 (Hennes & Mauritz, Far East) were used as feedstocks in this study. The cotton/PET
117 blending ratios were 80/20, 60/40 and 40/60. The cellulase producing fungal strain
118 *Aspergillus niger* CKB was obtained from Prof. Diannan Lu at Tsinghua University in
119 China.

120

121 2.2 Chemicals and reagents

122 Sodium citrate buffer (50 mM, pH 4.8) and 3,5-dinitrosalicylic acid (DNS) solution
123 were prepared according to the procedure illustrated by Adney and Baker (1996).
124 Citric acid monohydrate, Rochelle salt (potassium sodium tartrate) and 3,5-
125 dinitrosalicylic acid were purchased from Alfa Aesar (UK). Sodium hydroxide, potato
126 starch and lactose monohydrate were supplied by VWR BDH Prolabo (UK). Sucrose
127 (99.7%) and Avicel (cellulose microcrystalline, extra pure, a particle size of 90 μm)
128 were ordered from Acros Organics (Belgium). Carboxymethylcellulose (sodium salt)
129 and peptone were purchased from ChemCruz (USA) and UNI-CHEM (China),
130 respectively.

131

132 2.3 Solid state fermentation (SSF)

133 Fungal cellulase was produced on textile waste via solid state fermentation. For each
134 SSF, 2 g (dry weight) of textile waste scrap ($0.8 \times 0.8 \text{ cm}^2$) was mixed with 8 mL of
135 cultivation medium. The pH of the fermentation medium was adjusted to the
136 designated pH values (in the range of 4 - 8). After autoclaving at 121°C for 15 min,
137 the mixture was incubated with 0.3 mL of spore suspension (2×10^8 spores mL^{-1}) in a
138 petri dish. SSF was conducted in an incubator for 9 days under static condition. The
139 weight of each petri dish (with substrate and inoculum) was measured at the
140 beginning of SSF and was maintained constant throughout fermentation by addition
141 of deionization water (DI water) . Different temperature conditions ($25\text{-}35^\circ\text{C}$) and
142 initial moisture contents (55-95%) were employed. Each designed fermentation
143 condition was tested in duplicate to obtain parallel results.

144

145 2.4 Selection of cultivation medium

146 Three different cultivation media were compared in this study: (i) Csiszar medium
 147 (Csiszar *et al.* 2007), (ii) Mandels medium with peptone (Mendels and Weber, 1969)
 148 and (iii) Mandels medium with yeast extract (YE). The compositions of these three
 149 media are listed in Table 1.

150 Three types of cotton/PET blended textile fabric were separately mixed with the three
 151 cultivation media. After autoclaving, the mixture was incubated with *A. niger* CKB
 152 (3×10^7 spores g^{-1}) under 75% moisture, pH 6.0 for 9 days at 28°C in duplicate. At the
 153 end of SSF, all samples were collected for cellulase activity assay.

154 **Table 1.** Compositions of different cultivation media used in SSF on textile waste.

Substance (unit: $g L^{-1}$)	Csiszar medium	Mandels medium (with peptone)	Mandels medium (with YE)
Tween 80	-	1	1
Peptone	-	2.5 w/w%	
Yeast extract	2.5 w/w%	-	2.5 w/w%
Urea	-	0.3	0.3
KH_2PO_4	5	2	2
$(NH_4)_2SO_4$	-	1.4	1.4
NH_4NO_3	3	-	-
$(NH_4)_2HPO_4$	3	-	-
$MgSO_4$	0.5	0.3	0.3
NaCl	0.5	-	-
$CaCO_3$	0.5	-	-
$CaCl_2$	-	0.4	0.4
$FeSO_4$	-	0.005	0.005
$MnSO_4$	-	0.0016	0.0016
$ZnSO_4$	-	0.0014	0.0014
$CoCl_2$	-	0.002	0.002

155

156

157 2.5 Fungal cellulase extraction and assay

158 At the end of SSF, fungal cellulase was extracted and analysed. For each sample, 2 g
159 of fermented substrate was mixed with 60 mL of sodium citrate buffer (50 mM,
160 pH 4.8) in a blender (Ling Yang Frozen Machine Co., Hong Kong) for 10 seconds.
161 The mixture was then centrifuged at 4°C, 10,000 g for 3 minutes to collect the clear
162 supernatant as the crude enzyme sample. Total cellulase activity was determined by
163 the filter paper activity (FPase) using the standardised NREL Laboratory Analytical
164 Procedure (Adney and Baker, 1996).

165

166 In terms of the substrate used in this study, the FPase calculation was modified on the
167 basis of dry weight of textile (Eq. 1).

168

$$\text{FPase activity (FPU/g)} = \frac{\text{FPase activity (FPU/mL)} \times \text{Total volume of the fungal extract (mL)}}{\text{Dry weight of the textile waste used in SSF (g)}}$$

169

170 **Eq. (1)**

171

172

173 2.6 Optimisation of physical factors by Response Surface Methodology (RSM)

174 For optimisation of SSF conditions, four physical parameters including pH, yeast
175 extract concentration, inoculum size and moisture content were optimised via RSM
176 with a four-factor-five-level central composite design (CCD) by Design-Expert®
177 Software Version 8.0. Table 2 lists the value design of each factor. Cellulase activity
178 (FPase) was set as the single response.

179

180

181 **Table 2.** Central composition design of four factors on SSF for cellulase production.

Numeric factor	Unit	Low value	High value	-alpha	+alpha
(A) pH	-	5	7	4	8
(B) Yeast extract	w/w %	1	4	0	5.5
(C) Inoculum size	spores g ⁻¹	1.6E+007	4.6E+007	1E+006	6.1E+007
(D) Moisture	%	60	80	50	90

182

183 Based on the factors design above, a total of 30 runs with six centre points (set at the
184 middle-value of each factor) were suggested by the software. Two replicates of each
185 run were employed to verify any change in the estimation and experimental procedure.
186 Accordingly, 60 samples were prepared with the designed conditions and then
187 incubated for 9 days. To construct the model, all factors were coded using Eq. (2).

188

189
$$x_i = \frac{X_i - X_0}{\Delta X_i} \quad \text{Eq. (2)}$$

190 Where X_i and X_0 are actual values of an independent variable in non-centre points and
191 in centre points, respectively. The difference between X_i and X_0 was divided by step
192 change value ΔX_i to gain the dimensionless value x_i .

193 With the obtained experimental result, a second order polynomial equation was
194 suggested to describe the effect of each factor on cellulase production by linear,
195 quadratic and cross product terms, as presented in Eq. (3)

196

$$Y = a_0 + \sum_{i=1}^k a_{ij}X_i + \sum_{i=1}^k a_{ij}X_i^2 + \sum_i^k \sum_j^k a_{ij}X_i X_j + b$$

Eq. (3)

197

198

199 Where Y is cellulase activity as the single response, with i and j as linear coefficient
200 and quadratic coefficient, respectively. The letter “ a ” is a regression coefficient and
201 “ b ” is a random error. The number of factors is represented by “ k ”.

202

203 2.7 Statistical analysis

204 The influence of each fermentation condition on cellulase production was evaluated
205 by Analysis of Variance (ANOVA) based on F-test. Data processing and statistical
206 analysis were performed by the software Design-Expert® 8.0.

207

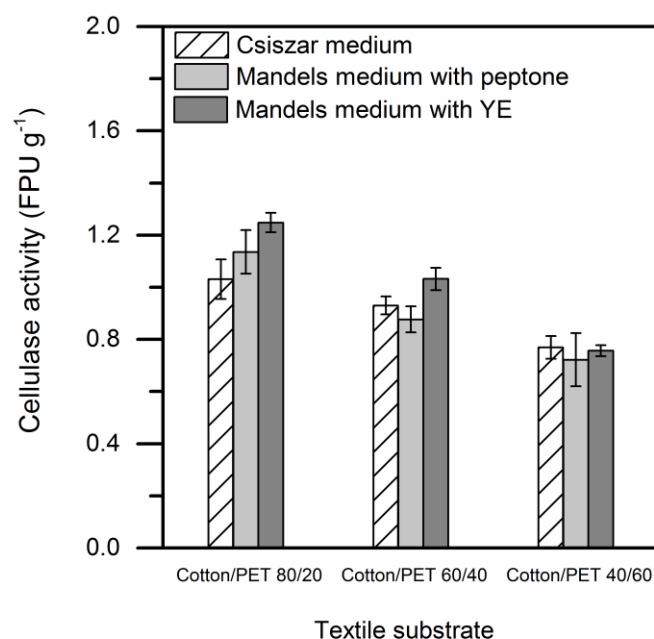
208 3. Results and discussion

209 3.1 Comparison of SSF on different cultivation media

210 The fermentation medium used in SSF usually consists of carbon and nitrogen sources,
211 phosphorus and mineral elements. Csiszar medium and Mandels medium are
212 commonly used for cellulase production using lignocellulosic substrate (Xin and
213 Geng, 2010). Cellulose in substrate serves as the essential carbon source to induce
214 cellulase generation, whereas nitrogen source can also stimulate cellulase and fungal
215 biomass production (Kachlishvili *et al.*, 2006). The effect of different cultivation
216 media on cellulase production varied outcomes with different substrates and fungal
217 strains (Kachlishvili *et al.*, 2006). Therefore, it is important to have a proper
218 combination of medium, substrate and fungal strain to maximise cellulase production

219 in SSF. In order to select a suitable medium for SSF on textile substrate using *A. niger*
220 CKB, Csiszar medium and Mandels medium with either peptone or yeast extract as
221 nitrogen source were investigated under the same incubation conditions.

222 As shown in Table 1, the main differences between Csiszar medium and Mandels
223 medium are their nitrogen sources, minerals and the presence of Tween 80. Peptone,
224 YE and ammonium salts are the commonly used nitrogen sources in fermentation
225 media. Kachlishvili *et al.* (2006) reported that peptone and $(\text{NH}_4)_2\text{SO}_4$ were the most
226 suitable nitrogen sources for CMCase production by *P. dryinus* on beech leaves and
227 wheat straw, respectively. In this study, peptone and yeast extract were separately
228 used as nitrogen sources in Mandels medium for SSF on textile. The result presented
229 in Figure 1 shows that the use of yeast extract as nitrogen source led to higher
230 cellulase activity on all three types of cotton/PET textile blends. In comparison to
231 Csiszar medium, Mandels medium (with YE) generated higher cellulase activity with
232 cotton/PET 80/20 and 60/40 blends as substrates. This could be attributed to the
233 acceleration by supplementary trace elements (Fe, Mn, Zn, Co), which are cofactors
234 of cellulase and supporting nutrients to fungal growth (Deswal *et al.*, 2011). Besides,
235 Tween 80 in Mandels medium acts as a surfactant to improve the permeability of
236 fungal cell membrane, which could enhance cellulase secretion (Ahamed and
237 Vermette, 2008). Accordingly, Mandels medium with YE as nitrogen source was
238 selected in SSF using textile waste, and the highest cellulase activity was 1.24 FPU g⁻¹
239 obtained from cotton/PET 80/20 blend.



240

241 **Figure 1.** Solid state fermentation on six types of textiles using various cultivation
 242 media.

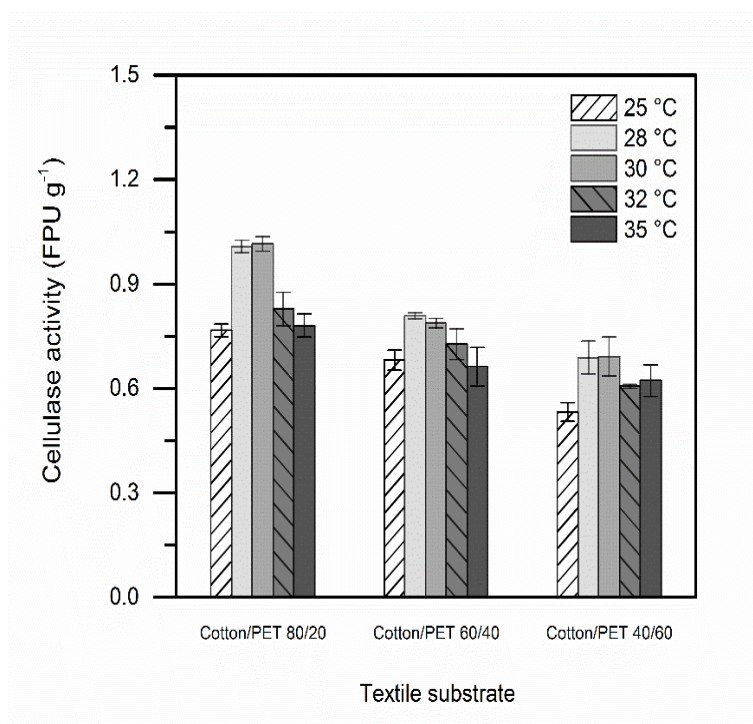
243

244 3.2 SSF under different temperature conditions

245 Incubation temperature is a fungal-dependent parameter that affects cellulase
 246 production in SSF. For instance, *Aspergillus sp.* cultivation on rice grass was
 247 suggested to be conducted at 32°C while *Fomitopsis sp.* was cultivated on wheat bran
 248 at 30°C (Deswal *et al.*, 2011; Liang *et al.*, 2012). In general, the optimal temperature
 249 for cellulase production by various fungi normally falls within a range of 25-34°C
 250 (Gautam *et al.*, 2011). According to the study of Javed and Khan (2006), 30°C was
 251 the optimised temperature for the fungal growth of *A. niger* species. In this study,
 252 three types of cotton/PET blended textile waste were used as substrates to explore the
 253 suitable temperature condition for cellulase production. *A. niger* CKB was cultivated
 254 on each type of textile at 25°C, 28°C, 30°C, 32°C and 35°C separately, with 75%

255 moisture and 3×10^7 spores g^{-1} inoculum size for 9 days. The result of cellulase
256 activities was determined as shown in Figure 2, from which the effect of different
257 temperatures on the three types of textile shows similar pattern. SSF at $25^\circ C$ limited
258 cellulase activity to a low level. As temperature raised to $28-30^\circ C$, the result was
259 significantly improved. Enzyme activities obtained at $28^\circ C$ and $30^\circ C$ were very
260 similar. Higher temperatures at $32-35^\circ C$ presented an obvious inhibition on cellulase
261 production. This is mainly because that higher temperature led to rapid loss of
262 moisture content from the fermentation substrate, hence the dry environment impaired
263 fungal growth and metabolic activity. The results pinpointed the optimal temperature
264 condition of $28^\circ C$ for cellulase production on textile waste.

265



266

267 **Figure 2.** Cellulase production on textile waste at different temperatures.

268

269

270 3.3 Optimisation via Response Surface Methodology

271 Results in Section 3.1-3.2 indicated that the highest cellulase activity was obtained
272 from 80/20 cotton/PET blend. Therefore, this textile was used as substrate in the
273 subsequent investigation. SSF conditions including pH, yeast extract concentration,
274 inoculum size and moisture content were optimised by RSM. Experiments were
275 conducted in 30 runs according to the four-factor-five-level Central Composite
276 Design (CCD). The specific condition of each run and the corresponding responses
277 are listed in Table 3. Six runs at the centre point were bolded.

278

279 **Table 3.** Results of CCD in RSM and the corresponding responses for optimisation of
 280 SSF conditions.

Run	pH	YE * (w/w %)	Inoculum size (spores g ⁻¹ textile)	Moisture (%)	Cellulase activity (FPU g ⁻¹)
1	7.00	4.00	4.60E+07	80.00	1.25
2	6.00	2.50	3.10E+07	70.00	1.24
3	4.00	2.50	3.10E+07	70.00	1.17
4	5.00	4.00	1.60E+07	60.00	0.73
5	6.00	2.50	1.00E+06	70.00	1.12
6	7.00	4.00	4.60E+07	60.00	0.85
7	6.00	5.50	3.10E+07	70.00	0.70
8	6.00	2.50	3.10E+07	70.00	1.25
9	7.00	1.00	4.60E+07	80.00	1.48
10	7.00	1.00	1.60E+07	60.00	0.76
11	6.00	2.50	3.10E+07	70.00	1.22
12	7.00	4.00	1.60E+07	60.00	0.61
13	6.00	2.50	3.10E+07	50.00	0.57
14	5.00	4.00	4.60E+07	60.00	0.91
15	8.00	2.50	3.10E+07	70.00	1.02
16	6.00	2.50	3.10E+07	70.00	1.32
17	7.00	1.00	4.60E+07	60.00	0.95
18	5.00	1.00	4.60E+07	60.00	0.78
19	6.00	2.50	3.10E+07	70.00	1.28
20	6.00	2.50	3.10E+07	70.00	1.33
21	6.00	2.50	6.10E+07	70.00	1.37
22	6.00	2.50	3.10E+07	90.00	1.04
23	5.00	1.00	1.60E+07	80.00	1.06
24	5.00	1.00	4.60E+07	80.00	1.05
25	5.00	4.00	1.60E+07	80.00	1.18
26	7.00	1.00	1.60E+07	80.00	1.08
27	7.00	4.00	1.60E+07	80.00	0.93
28	5.00	1.00	1.60E+07	60.00	0.75
29	5.00	4.00	4.60E+07	80.00	1.15
30	6.00	0.00	3.10E+07	70.00	0.57

281 *YE: yeast extract

282

283 The results show that cellulase activity obtained from cotton/PET 80/20 reduced over
 284 a range of 0.57-1.47 FPU g⁻¹. The condition in centre points resulted in cellulase
 285 activity of 1.22-1.33 FPU g⁻¹. Upon the result of response, a second-order polynomial
 286 model was constructed using Eq. (3) and Eq. (4). The ANOVA of each coded factor
 287 and interactions are listed in Table 4. The model F-value of 12.25 implies that the
 288 polynomial model was significant. There was statistically a 0.01% chance that this
 289 large F-value of model could occur due to noise. The effects of variables (A) pH, (B)
 290 yeast extract, (C) inoculum size and (D) moisture, along with interactions among
 291 these variables on cellulase activity were also evaluated by ANOVA.

292 **Table 4.** ANOVA of quadratic polynomial model for cellulase production from textile
 293 waste.

Source	Sum of squares	df	F-value	<i>p</i> -value Prob > F	Significance
Model	1.72	14	12.25	<0.0001	Significant
A	4.778E-006	1	0.00048	0.9829	
B	4.431E-003	1	0.44	0.5167	
C	0.14	1	12.68	0.0021	Significant
D	0.60	1	59.30	< 0.0001	Significant
AB	0.057	1	5.70	0.0305	Significant
AC	0.060	1	5.97	0.0274	Significant
AD	5.638E-003	1	0.56	0.4654	
BC	8.220E-004	1	0.082	0.7788	
BD	1.172E-004	1	0.012	0.9154	
CD	7.187E-005	1	0.0072	0.9337	
A ²	0.032	1	3.14	0.0969	
B ²	0.60	1	59.84	< 0.0001	Significant
C ²	1.127E-004	1	0.011	0.9171	
D ²	0.32	1	32.15	< 0.0001	Significant

294

295 The ANOVA results indicate that linear (C, D), interaction (AB, AC) and quadratic
 296 (B^2 , D^2) terms were statistically significant with p -values less than 0.0500. It means
 297 that these terms are the main affecting factors in regard to cellulase activity from SSF
 298 on textile waste. The model equation in terms of actual value of variables and
 299 response is described in Eq. (4).

300

$$\begin{aligned}
 301 \quad & \text{Cellulase activity} = - 6.0143 + 0.24656 \times \text{pH} + 0.62448 \times \text{YE} - 2.17814 \times 10^{-8} \times \\
 302 \quad & \text{Inoculum ratio} + 0.15584 \times \text{Moisture} - 0.0399 \times \text{pH} \times \text{YE} + 4.08083 \times 10^{-9} \times \text{pH} \times \\
 303 \quad & \text{Inoculum ratio} + 1.87716 \times 10^{-3} \times \text{pH} \times \text{Moisture} + 3.1856 \times 10^{-10} \times \text{YE} \times \text{Inoculum} \\
 304 \quad & \text{ratio} - 1.80423 \times 10^{-4} \times \text{YE} \times \text{Moisture} + 1.41297 \times 10^{-11} \times \text{Inoculum ratio} \times \text{Moisture} \\
 305 \quad & - 0.033764 \times \text{pH}^2 - 0.074587 \times \text{YE}^2 + 8.97195 \times 10^{-18} \times \text{Inoculum ratio}^2 - \\
 306 \quad & 1.08093 \times 10^{-3} \times \text{Moisture}^2
 \end{aligned}$$

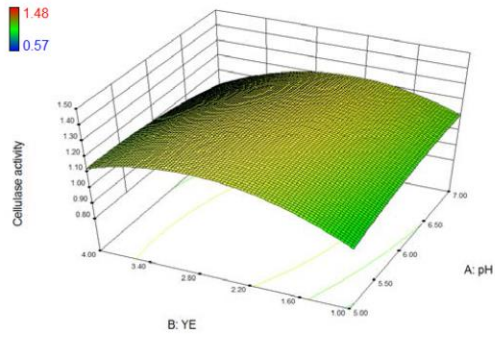
307 **Eq. (4)**

308

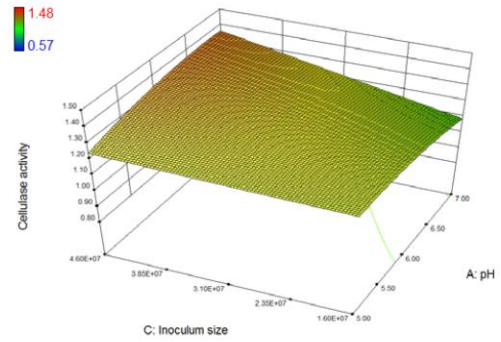
309 The coefficient of determination (denoted as R^2) of the equation was 0.9196 and the
 310 adjusted R^2 was 0.8445, indicating that the fitted linear, interaction and quadratic
 311 terms could elucidate 84.45% of the variation. The signal-to-noise ratio was measured
 312 by Adequate Precision, which is desired to be higher than 4. The constructed model
 313 ratio of 12.795 indicated an adequate signal so that the model can be used to navigate
 314 the design space.

315 The interactions between any two variables were depicted by response surface plots
 316 (3D) in Figures 3, with other factors fixed at the centre point values. Figure 3a shows
 317 the response surface curve of the resultant cellulase activity in response to changes in
 318 pH and yeast extract concentration, while the initial moisture content and inoculum
 319 size were maintained at 70% and 3.1×10^7 spore g^{-1} , respectively. As shown in
 320 Figure 3a, cellulase activity reached a maximum at pH 5.5-6.5 and yeast extract
 321 concentration of 2.20-3.40 w/w%. Figure 3b shows the significant interaction between

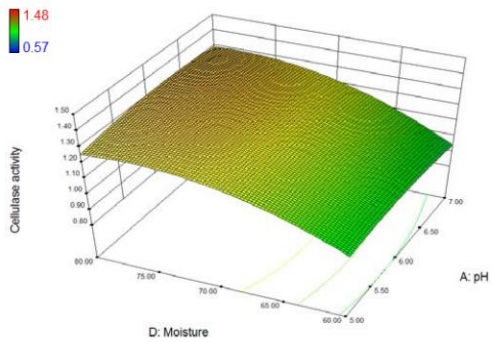
322 pH and inoculum size. As inoculum size and pH increased, cellulase activity grew
323 from 1.10 to 1.30 FPU g⁻¹. In contrast, low cellulase activity was obtained at limited
324 inoculum size (1.60×10^7 spore g⁻¹) and high pH value (> 7.0). At high levels of
325 inoculum size, pH range of 5.5-7.0 slightly affected cellulase activity. While under
326 neutral pH conditions (6.5-7.0), cellulase activity were sensitive to inoculum size. The
327 response surface curve in Figure 3c indicates that the interactive effect of moisture
328 and pH was obviously dominated by the former. When yeast extract concentration
329 was 2.5 w/w% at centre point value, cellulase activity was gradually improved with
330 increase of inoculum size (Figure 3d). Figure 3e shows an optimum response was
331 reached under slightly higher moisture conditions (75-80%) in the interaction with
332 yeast extract. Decrease of cellulase production occurred at both low and high levels of
333 yeast extract, because of nutrient depletion and the inhibitory effect of nutrient surplus,
334 respectively. Besides, although inoculum size and moisture both exhibited profound
335 positive effects on cellulase production, the interaction was insignificant ($p > 0.05$). In
336 spite of this, it could be noted from Figure 3f that the high levels of moisture (75-80%)
337 and inoculum size ($> 3.1 \times 10^7$ spore g⁻¹) enhanced cellulase activity to the peak value.



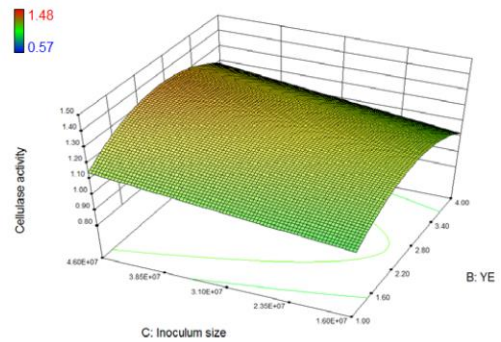
(a) Interaction between pH and yeast extract



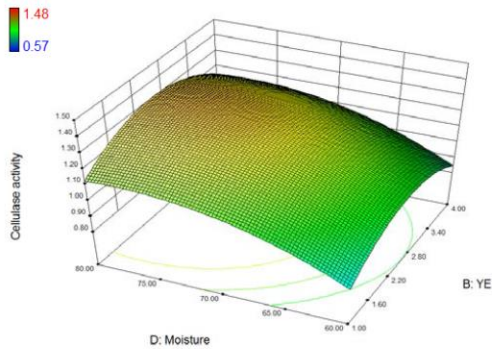
(b) Interaction between pH and inoculum size



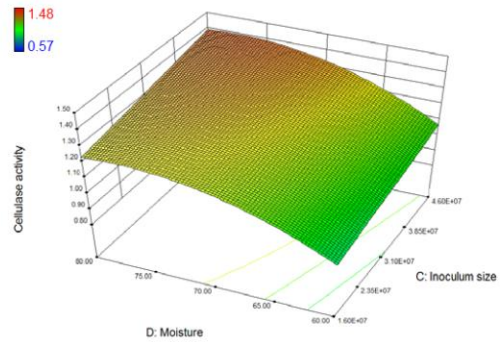
(c) Interaction between pH and moisture



(d) Interaction between YE and inoculum size



(e) Interaction between YE and moisture



(f) Interaction between inoculum size and moisture

338

339

Figure 3. Response surface 3D plots of the interactions between various examined

340

fermentation conditions.

341

342 In summary, the model suggested the optimum SSF condition at pH 7.29, yeast
343 extract 2.24 w/w% and moisture of 78.53% with inoculum size of 4.60×10^7 spore g^{-1}
344 for the maximised cellulase activity within the fixed range of each variable.
345 Accordingly, cellulase activity of 1.48 FPU g^{-1} was predicted. Hence, the suggested
346 condition was tested with different inoculum sizes of 1.60×10^7 , 3.10×10^7 and
347 4.60×10^7 spore g^{-1} , resulting in cellulase activities of 1.13, 1.44 and 1.46 FPU g^{-1} ,
348 respectively. This revealed that under suitable conditions, adequate inoculum size is
349 essential to high cellulase activity. However, this could not be linearly improved with
350 further increase of inoculum size.

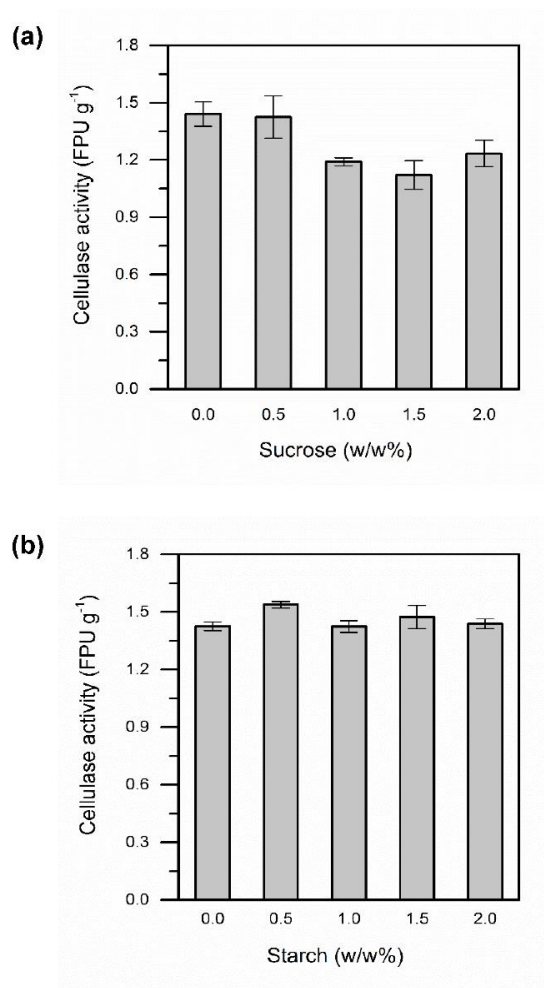
351 Therefore, based on the results above and by considering the experimental feasibility,
352 the optimum SSF condition for cellulase production from textile waste was suggested
353 to be pH 7.29, 2.24 w/w% yeast extract with a moisture content of 78% and an
354 inoculum size of 3.10×10^7 spore g^{-1} . Fungal cellulase activity was improved by 16-
355 20% from 1.20-1.24 FPU g^{-1} to 1.44 FPU g^{-1} .

356

357 3.4 Cellulase activity improvement by supplementary carbon sources

358 In order to improve fungal cellulase activity, sucrose and starch were added as
359 supplementary carbon sources to enhance the fungal growth and enzyme production.
360 Different loading ratios (0, 0.5, 1.0, 1.5, 2.0 w/w%) of sucrose/starch were added in
361 SSF medium on the textile of cotton/PET 80/20. The optimum incubation conditions
362 (from Section 3.2-3.3) of 9 days were applied in duplicate. The resultant cellulase
363 activity is depicted in Figure 4.

364



365

366 **Figure 4.** Effect of supplementary (a) sucrose and (b) starch on cellulase production

367

from textile waste.

368

369 The results reveal that the addition of sucrose did not improve cellulase activity.

370 Addition of sucrose as an inducer at relatively higher loading ratios (1.0-2.0 w/w%)

371 even exhibited an inhibitory effect on cellulase production. In comparison,

372 supplementary starch enhanced cellulase activity to a certain extent. The significance

373 of variances caused by different starch dosages was judged by ANOVA (Table 5).

374 The addition of starch by 0.5, 1.5 and 2.0 w/w% led to a positive effect on cellulase

375 activity. ANOVA result pointed out that when 0.5 w/w% starch was added, the
 376 corresponding cellulase activity improvement (from 1.43 to 1.53 FPU g⁻¹) was
 377 significant as *p*-value was lower than 0.05. In contrast, variances caused by other
 378 loading ratios could have occurred due to noise with high probability (40.24% -
 379 98.69%). Therefore, the supplement of 0.5 w/w% starch was suggested in SSF for
 380 cellulase production from textile waste.

381 **Table 5.** Effect of supplementary starch on cellulase production from textile waste by
 382 ANOVA.

Starch addition (w/w%)	Cellulase activity (FPU g ⁻¹)	Standard deviation	F value	<i>p</i> value Prob > F
0.0	1.43	0.0212	N/A	N/A
0.5	1.53	0.0170	35.22	0.0272
1.0	1.42	0.0318	3.42E-04	0.9869
1.5	1.47	0.0608	1.11	0.4024
2.0	1.44	0.0490	0.31	0.6348

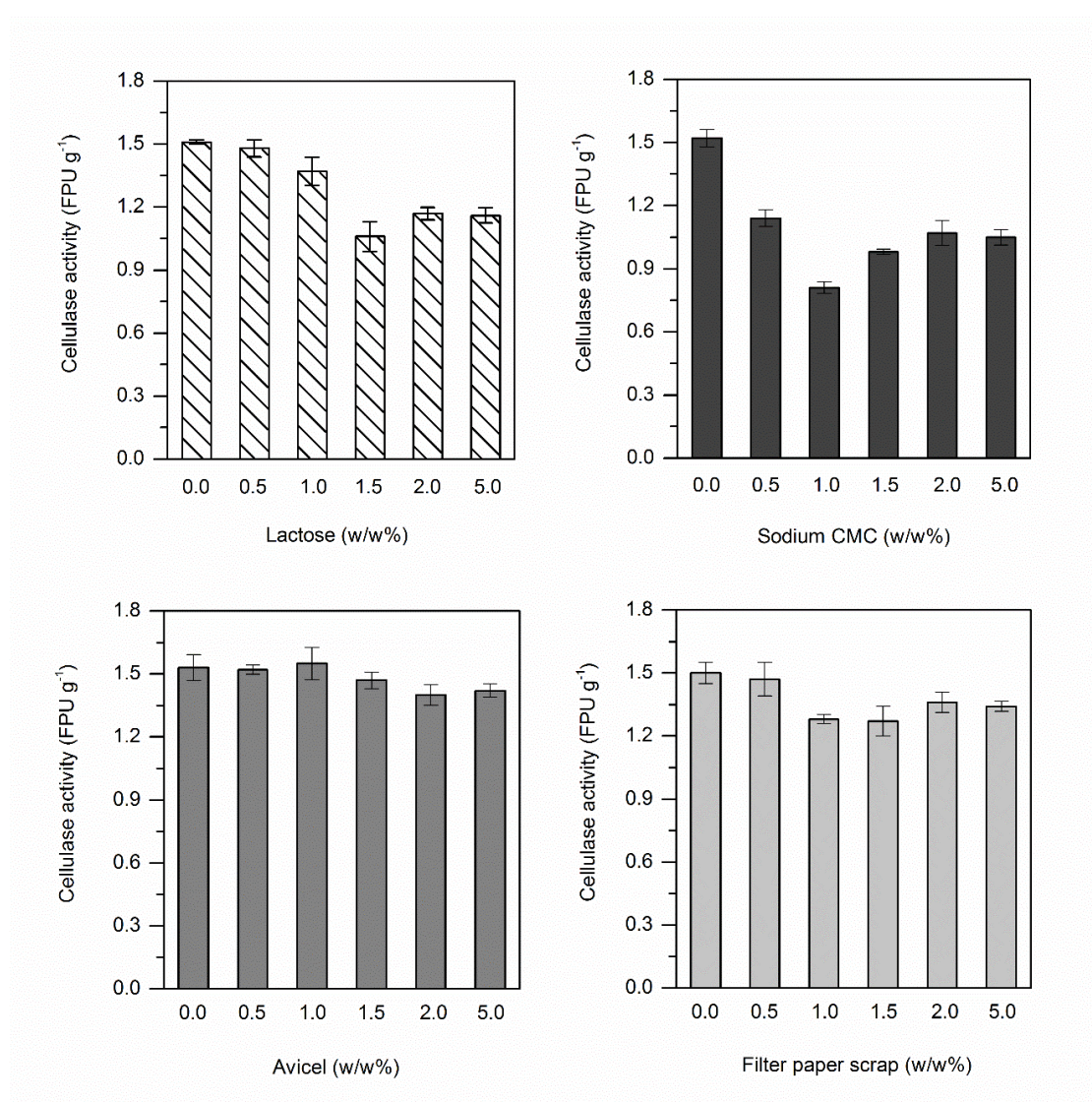
383

384

385 3.5 Effect of inducer on cellulase production from textile waste

386 Natural inducers of fungal cellulase generation have been investigated since 1957 in
 387 Mandel and Reese's study (Mandels and Reese, 1957). They suggested that cellulase
 388 could only be produced on glucose, lactose, cellubiose and cellulose. Lactose and
 389 basic celluloses consisting of anhydroglucose units with β -1-4-glycosidic linkage
 390 were proposed as excellent inducers to stimulate cellulase secretion towards breaking
 391 β -1-4-glycosidic bonds to obtain the monomeric glucose. Therefore, lactose has been
 392 applied as an inducer in the commercial production of cellulase (Sukumaran *et al.*,
 393 2005).

394 In this study, lactose and basic celluloses including Avicel, sodium carboxymethyl
395 cellulose (sodium CMC) and filter paper scrap (Whatman No.1, 100% cellulose) were
396 employed as inducers in SSF on textile waste. Inducers were added separately in
397 gradients from 0.5 to 5.0 w/w%. SSF was performed on cotton/PET 80/20 blend using
398 the optimum condition with 0.5 w/w% starch for 9 days. A control group was set
399 without any inducer. All conditions were conducted in duplicate and the harvested
400 cellulase activities are depicted in Figure 5.



401

402 **Figure 5.** Effect of inducers on cellulase production from textile waste

403

(80/20 cotton/PET blend).

404

405 The results show that the addition of lactose, sodium CMC or filter paper scraps failed
406 to enhance cellulase activity to a higher level. In these three sets, the highest cellulase
407 activities were obtained from the control group (without inducer). Sodium CMC
408 loaded at weight ratios higher than 1% exhibited significant inhibitory effect on
409 cellulase production, resulting in a reduction of enzyme activity from 1.52 FPU g⁻¹ to
410 0.81-1.07 FPU g⁻¹. In comparison, with 1.0 w/w% of Avicel as an inducer, cellulase
411 activity increased slightly from 1.53 to 1.55 FPU g⁻¹.

412 The insignificant inducing effect could be attributed to several possible reasons.
413 Firstly, the metabolic activity of *A. niger* CKB cannot be simply induced through
414 direct addition of Avicel/basic cellulose into fermentation medium. Secondly, the
415 heterogeneous substrate by mixing insoluble inducers (*e.g.* sodium CMC, filter paper
416 scraps) with textile fabric is not suitable for fungal growth. For instance, the addition
417 of sodium CMC in textile substrate led to high viscosity of mixture and thereby
418 inhibited aerobic condition along with fungal colonisation. Besides, the time course of
419 cellulase production might have been affected by supplementary starch and the
420 inducer. Therefore, the enzyme activity collected on day 9 could not clearly
421 distinguish the variance.

422

423 3.6 Time course of cellulase activity under optimised SSF conditions

424 The time course of fungal cellulase production from textile waste was investigated in
425 our previous study (Hu *et al.*, 2017). The total cellulase activity increased from day 3
426 and reached peak value on day 9 in SSF. In this study, the cultivation conditions were
427 optimised, in which it essentially affected the cellulase activity of fungal enzyme
428 product. Thus, the corresponding time course deserves further exploration in order to

429 determine the production profile of cellulase activity. *A. niger* CKB was incubated on
430 the textile of cotton/PET 80/20 under different conditions in Set A, B and C as listed
431 in Table 6.

432 **Table 6.** Fungal cellulase production under different SSF conditions.

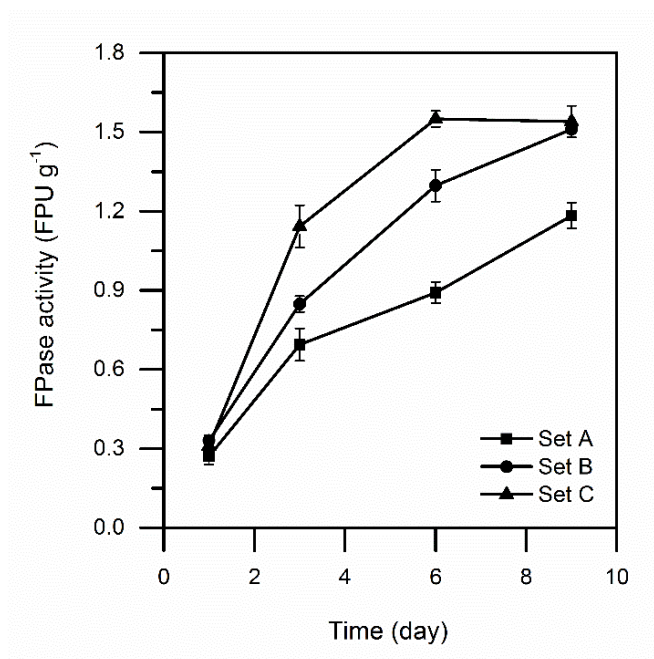
	Set A	Set B	Set C
Moisture condition (%)	75.0	78.0	78.0
Inoculum size (10^7 spores g^{-1})	3.1	3.1	3.1
pH	6.0	7.2	7.2
Yeast extract (w/w%)	2.50	2.24	2.24
Starch (w/w%)	0.0	0.5	0.5
Avicel (w/w%)	0.0	0.0	1.0

433

434 The conditions applied in Set A are the middle-value of each factor, without
435 additional carbon source or inducer. Conditions in Set B were the optimal solution
436 from RSM according to the results in Section 3.3 and with starch (0.5 w/w%) as a
437 supplementary carbon source. Furthermore, Avicel (1.0 w/w%) was supplied as an
438 inducer in Set C. All sets were tested in duplicate at 28°C. Figure 6 shows the
439 cellulase activity (FPase) profile of Set A, B and C.

440 It was found that under optimal SSF conditions, cellulase activity in Set B and C
441 increased from day 1 at higher efficiency as compared to the status in Set A. The
442 addition of Avicel (in Set C) further improved cellulase activity and led to the
443 maximum of 1.56 FPU g^{-1} on day 6. Therefore, the combination of optimum
444 fermentation conditions, supplementary starch and Avicel indeed enhanced fungal
445 cellulase production, which was consequently accomplished in reduced incubation
446 period (from 9 days to 6 days) with 25.8% increase of total cellulase activity. The

447 production efficiency of fungal cellulase from textile waste was significantly
448 improved by 88.7%.



449

450 **Figure 6.** Time courses of cellulase production under different SSF conditions.

451

452 Upon the optimum SSF, the overall process of the novel circular textile waste
453 approach in the project entitled “Textile Waste Recycling by Biological Method” is
454 described in Figure 7. Textile waste from manufacturers and from fermented substrate
455 in SSF was inputted as raw materials. The fungal cellulase (1.56 FPU g⁻¹) was
456 employed as an enzyme source in textile waste hydrolysis, which led to a glucose
457 recovery yield of 70.2% within 96 h. The resultant hydrolysate was a glucose-rich
458 stream separated from the remaining solid (*i.e.* polyester) by filtration. This glucose-
459 rich stream could be utilised as a generic feedstock in microbial fermentation for the
460 production of value-added products via product refining. According to the
461 experimental results, around 624 kg of glucose and 200 kg of PET could be recovered
462 from 1,000 kg of textile waste (cotton/PET 80/20). The recovered PET was processed
463 into fibres by melting spinning towards textile applications for the first time by Li *et*

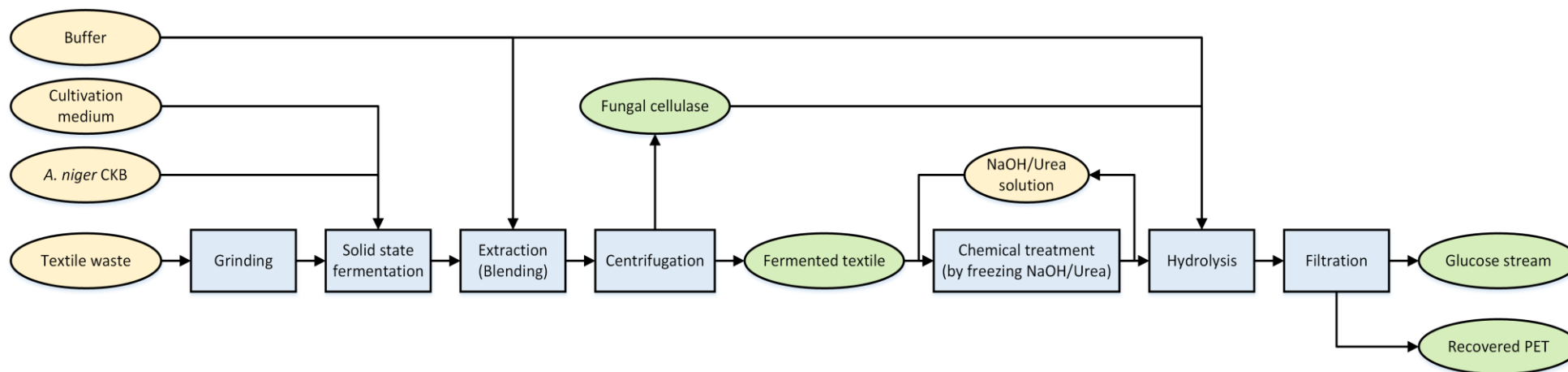
464 *al.* As compared to recovering PET merely by paper filtration (Shen *et al.*, 2013;
465 Jeihanipour *et al.*, 2010), melting spinning waste PET into textile fibre would
466 definitely increase its commercial value. Therefore, through the proposed biorefining
467 approach, textile waste can be efficiently recycled to value-added products, which can
468 benefit the circular economy. The economic performance and technical feasibility of
469 the overall process at pilot scale is currently under evaluation in our group.

470

471 **4. Conclusions**

472 This study illustrated the optimisation of fungal cellulase production from textile
473 waste using experimental design. Different cultivation media were compared on three
474 types of cotton/PET blended textile waste. Typical fermentation affecting factors were
475 optimised through one-variable method and Response Surface Methodology, which
476 suggested the optimum SSF conditions by using Mandels medium with yeast extract
477 (2.24 w/w%), the moisture content of 78%, the inoculum size of 3.10×10^7 spore g^{-1}
478 and pH 7.29 at 28°C. The addition of starch (0.5 w/w%) and Avicel (1 w/w%) further
479 increased cellulase activity to 1.56 FPU g^{-1} with significantly improved production
480 efficiency. The outcomes reported in this study could contribute to an innovative
481 circular textiles approach, which enables the transition from the current linear to
482 stronger circular economy model.

483



484

485

486

Figure 7. Process scheme of the textile waste recycling approach through biological method.

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496

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499 **References**

- 500 Adney, B., Baker, J., 1996. Measurement of cellulase activities. Laboratory Analytical
501 Procedure, **6**, 1996.
- 502 Ahamed, A., Vermette, P., 2008. Culture-based strategies to enhance cellulase
503 enzyme production from *Trichoderma reesei* RUT-C30 in bioreactor culture
504 conditions. Biochem. Eng. J. **40**(3), 399-407.
- 505 Bansal, N., Tewari, R., Soni, R., Soni, S.K., 2012. Production of cellulases from
506 *Aspergillus niger* NS-2 in solid state fermentation on agricultural and kitchen
507 waste residues. Waste Manage. **32**(7), 1341-1346.
- 508 Caulfield, K., 2009. Sources of textile waste in Australia. Apical International,
509 Australia.
510 [http://www.nacro.org.au/wp-content/uploads/2013/04/TEXTILE-WASTE-](http://www.nacro.org.au/wp-content/uploads/2013/04/TEXTILE-WASTE-PAPER-March-2009-final.pdf)
511 [PAPER-March-2009-final.pdf](http://www.nacro.org.au/wp-content/uploads/2013/04/TEXTILE-WASTE-PAPER-March-2009-final.pdf) (Accessed on June 08, 2017)
- 512 Csiszar, E., Szakacs, G., & Koczka, B., 2007. Biopreparation of cotton fabric with
513 enzymes produced by solid-state fermentation. Enzyme Microb. Technol.,
514 **40**(7), 1765-1771.
- 515 Deswal, D., Khasa, Y.P., Kuhad, R.C., 2011. Optimization of cellulase production by
516 a brown rot fungus *Fomitopsis sp.* RCK2010 under solid state fermentation.
517 Bioresour. Technol. **102**(10), 6065-6072.
- 518 EPD., 2015. Monitoring of Solid Waste in Hong Kong: Waste Statistics for 2014.
519 Environmental Protection Department.
520 <https://www.wastereduction.gov.hk/sites/default/files/msw2014.pdf> (Accessed
521 on June 08, 2017)
- 522 Gautam, S., Bundela, P., Pandey, A., Khan, J., Awasthi, M., Sarsaiya, S., 2011.
523 Optimization for the production of cellulase enzyme from municipal solid
524 waste residue by two novel cellulolytic fungi. Biotechnol. Res. Int. **2011**.
- 525 Hu, Y., Du, C., Li, X., Lin, C.S.K., 2018. Circular textile waste-based biorefinery
526 development: Valorisation of textile waste for fungal cellulase production by
527 solid state fermentation. Resour. Conserv. Recycl., **129**, 27-35.
- 528 H&M, 2017. The H&M group Sustainability Report 2016.
529 [https://sustainability.hm.com/content/dam/hm/about/documents/en/CSR/Repor](https://sustainability.hm.com/content/dam/hm/about/documents/en/CSR/Report%202016/HM_group_SustainabilityReport_2016_CircularAndRenewable_en.pdf)
530 [t%202016/HM_group_SustainabilityReport_2016_CircularAndRenewable_en](https://sustainability.hm.com/content/dam/hm/about/documents/en/CSR/Report%202016/HM_group_SustainabilityReport_2016_CircularAndRenewable_en.pdf)
531 [.pdf](https://sustainability.hm.com/content/dam/hm/about/documents/en/CSR/Report%202016/HM_group_SustainabilityReport_2016_CircularAndRenewable_en.pdf) (Accessed on July 08, 2017)

532 Javed, M.M., Khan, T.S., 2006. An innovative approach for hyperproduction of
533 cellulolytic and hemicellulolytic enzymes by consortium of *Aspergillus niger*
534 MSK-7 and *Trichoderma viride* MSK-10. Afr. J. Biotechnol. **5**(8), 609-614.

535 Jeihanipour, A., Karimi, K., Niklasson, C., Taherzadeh, M.J., 2010. A novel process
536 for ethanol or biogas production from cellulose in blended-fibers waste textiles.
537 Waste Manage. **30**(12), 2504-2509.

538 Kachlishvili, E., Penninckx, M.J., Tsiklauri, N., Elisashvili, V., 2006. Effect of
539 nitrogen source on lignocellulolytic enzyme production by white-rot
540 *basidiomycetes* under solid-state cultivation. World J. Microbiol. Biotechnol.
541 **22**(4), 391-397.

542 Kumar, S., Sharma, H., Sarkar, B., 2011. Effect of substrate and fermentation
543 conditions on pectinase and cellulase production by *Aspergillus niger* NCIM
544 548 in submerged (SmF) and solid state fermentation (SSF). Food Sci.
545 Biotechnol. **20**(5), 1289-1298.

546 Lenzing Corporation. 2016. The global fibre market.
547 <http://www.lenzing.com/en/fibers/the-global-fiber-market.html> (Accessed on
548 June 08, 2017)

549 Levin, L., Herrmann, C., Papinutti, V.L., 2008. Optimization of lignocellulolytic
550 enzyme production by the white-rot fungus *Trametes trogii* in solid-state
551 fermentation using response surface methodology. Biochem. Eng. J. **39**(1),
552 207-214.

553 Li, X., Hu, Y., Du, C., Lin, C.S.K. Recovery of glucose and polyester from textile
554 waste by enzymatic hydrolysis. Waste Biomass Valori. (under review)

555 Liang, X., Huang, Y., Hua, D., Zhang, J., Xu, H., Li, Y., Zhang, X., 2012. Cellulase
556 production by *Aspergillus sp.* on rice grass (*Spartina spp.*) under solid-state
557 fermentation. Afr. J. Microbiol. Res. **6**(39), 6785-6792.

558 Mandels, M., Reese, E.T., 1957. Induction of cellulase in *Trichoderma viride* as
559 influenced by carbon sources and metals. J. Bacteriol. **73**(2), 269.

560 Mendels, M., Weber, J., 1969. The production of cellulases. Adv. ChemSer. **95**, 395-
561 414.

562 Olsson, L., Christensen, T.M., Hansen, K.P., Palmqvist, E.A., 2003. Influence of the
563 carbon source on production of cellulases, hemicellulases and pectinases by
564 *Trichoderma reesei* Rut C-30. Enzyme Microb. Technol. **33**(5), 612-619.

565 Orzua, M.C., Mussatto, S.I., Contreras-Esquivel, J.C., Rodriguez, R., de la Garza, H.,
566 Teixeira, J.A., Aguilar, C.N., 2009. Exploitation of agro industrial wastes as
567 immobilization carrier for solid-state fermentation. *Ind. Crops Prod.* **30**(1), 24-
568 27.

569 Pensupa, N., Leu, S.Y., Jing, H., Liu, H., Hu, Y., Wang, H., Du, C., Lin, C.S.K., 2017.
570 Recent trends in sustainable textile waste recycling methods: current situation
571 and future prospects. *Top. Curr. Chem.* **375**, 76.

572 Shen, F., Xiao, W., Lin, L., Yang, G., Zhang, Y., Deng, S., 2013. Enzymatic
573 saccharification coupling with polyester recovery from cotton-based waste
574 textiles by phosphoric acid pretreatment. *Bioresour. Technol.* **130**, 248-255.

575 Soleimaninanadegani, M., Madihah, M., Ang, S., 2014. Factors affecting cellulase
576 production by *Aspergillus fumigatus* SK1 from solid state fermentation of oil
577 palm empty fruit bunches using application of 2-level factorial design.
578 *Bulletin of Environ. Sci. Res.* **3**(2-3), 16-24.

579 Sukumaran, R.K., Singhanian, R.R., Pandey, A., 2005. Microbial cellulases-production,
580 applications and challenges. *J. Sci. Ind. Res.* **64**, 832-844.

581 Sweeny, G., 2015. Fast Fashion Is the Second Dirtiest Industry in the World, Next to
582 Big Oil.
583 [https://www.ecowatch.com/fast-fashion-is-the-second-dirtiest-industry-in-the-](https://www.ecowatch.com/fast-fashion-is-the-second-dirtiest-industry-in-the-world-next-to-big--1882083445.html)
584 [world-next-to-big--1882083445.html](https://www.ecowatch.com/fast-fashion-is-the-second-dirtiest-industry-in-the-world-next-to-big--1882083445.html) (Accessed on July 08, 2017)

585 Textile Exchange. 2012. FastFacts: textile and product waste.
586 <http://www.purewaste.org/media/pdf/textile-product-waste-fast-facts.pdf>.
587 (Accessed on June 08, 2017)

588 Ustok, F.I., Tari, C., Gogus, N., 2007. Solid-state production of polygalacturonase by
589 *Aspergillus sojae* ATCC 20235. *J. Biotechnol.* **127**(2), 322-334.

590 WRAP., 2016. Textiles Market Situation Report. Waste & Resources Action
591 Programme..
592 [http://www.wrap.org.uk/sites/files/wrap/Textiles_Market_Situation_Report_2](http://www.wrap.org.uk/sites/files/wrap/Textiles_Market_Situation_Report_2016.pdf)
593 [016.pdf](http://www.wrap.org.uk/sites/files/wrap/Textiles_Market_Situation_Report_2016.pdf). (Accessed on Mar 03, 2017)

594 Xin, F., Geng, A., 2010. Horticultural waste as the substrate for cellulase and
595 hemicellulase production by *Trichoderma reesei* under solid-state
596 fermentation. *Appl. Biochem. Biotechnol.* **162**(1), 295-306.

597 Yasmeen, Q., Asgher, M., Sheikh, M.A., Nawaz, H., 2013. Optimization of
598 ligninolytic enzymes production through response surface methodology.
599 *Bioresources*, **8**(1), 944-968.
600 Yoon, L.W., Ang, T.N., Ngoh, G.C., Chua, A.S.M., 2014. Fungal solid-state
601 fermentation and various methods of enhancement in cellulase production.
602 *Biomass Bioenergy*, **67**, 319-338.
603