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Beta-Glucosidase From *Streptomyces Griseus*: Nanoparticle Immobilisation and Alkyl Glucoside Synthesis.

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1 **β -Glucosidase from *Streptomyces griseus*: nanoparticle**
2 **immobilisation and alkyl glucoside synthesis.**

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17 **ABSTRACT**

18 A novel β -glucosidase from *Streptomyces griseus* was cloned and overexpressed in
19 *E. coli*. The purified β -glucosidase (44 kDa) had a K_m of 8.6 ± 0.5 mM and a V_{max} of
20 217 ± 5.0 $\mu\text{moles}^{-1}\text{min}^{-1}\text{mg}$ at 37 °C, pH 7.2 with *p*-nitrophenyl- β -D glucopyranoside
21 as substrate. The enzyme was characterised in terms of pH optimum (pH 6.9),
22 temperature optimum (69 °C) and the influence of solvents and effectors. Purified *S.*
23 *griseus* β -glucosidase was successfully immobilised, by simple absorption, onto zinc
24 oxide (ZnO) nanoparticles without covalent modification. It remained tightly bound
25 even after extensive washing and could be reused up to ten times without significant
26 loss of activity. The immobilised enzyme had a higher optimum temperature and
27 greater thermostability than the free enzyme. In immobilised form the enzyme
28 readily catalysed the synthesis of alkyl glucosides.

29

30

31

32 **Keywords:** β -glucosidase, *Streptomyces griseus*, nanoparticle, alkyl glucoside,
33 immobilisation

34

35 **List of abbreviations:** ORF, Open Reading Frame; GST, Glutathione S Transferase;
36 NCBI, National Centre for Biotechnology Information; GYM, Glucose Yeast Malt
37 medium; *bglG*, β -glucosidase from *Streptomyces griseus*; pNGP, para nitrophenyl-
38 β -D glucopyranoside.
39

40 **Introduction**

41

42 β -glucosidases (*bglG*, EC 3.2.1.21) are hydrolytic enzymes that cleave β -glycosidic
43 bonds of carbohydrates [1], [2]. They act on a broad range of β -glycosides and play
44 important biological roles in both eukaryotic and prokaryotic organisms. They play a
45 particularly key role in the degradation of celluloses [3]. β -glucosidases have been
46 studied from many different living organisms such as marine invertebrates, bacteria,
47 fungi, plants, and mammals [4]. Commercially, they are used in the food and
48 pharmaceutical industries, chemicals production, textiles and in the biotechnology
49 sector. Indeed, β -glucosidases are amongst the most widely used enzymes for
50 biotechnological applications [5],[6].

51

52 Chemical synthesis using β -glucosidases as a biocatalyst is a significant area of
53 research interest. Such chemical transformations may involve transglycosylation or
54 reverse hydrolysis reaction pathways. Reverse hydrolysis involves the direct
55 esterification of a glycosyl donor and an acceptor. Thus, β -glucosidases have been
56 used to synthesise alkyl, flavanoid, stilbenoid and vitamin glycosides among others
57 (see [7] for review). Such transformations are examples of green chemistry that
58 minimize the use of organic solvents.

59

60 In many cases β -glucosidases for use in industrial processes are immobilised on a
61 solid support. Immobilisation of an enzyme catalyst allows for its separation from
62 reactants, reusability, and often increases its thermostability [8]. β -glucosidases have
63 been immobilized on different supports, such as alginate [9], silica gel [10], magnetic
64 chitosan microspheres [11], Eupergit C [12], magnetic nanoparticles [13] and, most
65 recently, silicone polymeric thin films [14]. A significant drawback with
66 immobilisation is that it often requires covalent attachment of the enzyme to a
67 support using crosslinking agents such as glutaraldehyde or carbodiimide. The
68 process of immobilization is time consuming, expensive and may lead to loss of
69 enzyme activity (see [15]).

70

71 In this study, a putative nucleotide sequence of a β -glucosidase was selected from the
72 *Streptomyces griseus* subsp. *griseus* genome database. Several β -glucosidase Open
73 Reading Frames ranging from 406 to 768 amino acids were identified in the genome

74 of *Streptomyces griseus subsp. griseus* using the NCBI genomic database. The
75 smallest ORF having a size of 406 amino acids was selected since it was thought that
76 a smaller protein might be more compact in structure and therefore more solvent and
77 temperature tolerant. Overexpression of this β -glucosidase was carried out under the
78 inducible control of a *tac* promoter based *Glutathione-S-Transferase* (GST) fusion
79 protein expression system to avoid the formation of inclusion bodies. A simple
80 method to immobilise this enzyme on zinc nanoparticles was developed and the
81 application of this enzyme in the synthesis of alkyl glucosides was demonstrated.

82

83

84 **Materials and Methods**

85

86 **Materials:**

87 Wizard Genomic DNA Purification Kit (Cat. No. A1125), Pure Yield™ Plasmid
88 Midi Prep System (Cat. No. A9281), Gel extraction kit, 6x DNA loading buffer, T4
89 DNA ligase, and GoTaq® DNA polymerase were obtained from Promega, MSC,
90 Dublin, Ireland. Gene specific primers were obtained from Eurofins MWG Operon.
91 Restriction endonucleases and their corresponding buffers were obtained from New
92 England Biolabs (NEB, UK). Glutathione Sepharose 4B resin and PreScission
93 protease were obtained from VWR International Ltd. Blanchardstown, Dublin,
94 Ireland. All chemicals were obtained from Sigma-Aldrich, Ireland.

95

96 **Bacterial strains, culture media, and growth conditions:**

97 *Streptomyces griseus* subsp. *griseus* was inoculated from stock culture into GYM
98 *Streptomyces* medium and incubated for three days at 220rpm and 37°C. Luria-
99 Bertani media was used for the maintenance of *E. coli* strains at 37°C. *Escherichia*
100 *coli* strains; JM109 (Promega) and BL21 (DE3) cells were used for cloning and
101 protein expression respectively. Phenotypically, protease-plus *E. coli* (JM109) was
102 used for maintenance of the vector pGEX-4T1 and *S. griseus* β -glucosidase (*pGST-*
103 *bglG*) clones.

104

105 **Amplification of β -glucosidase gene:**

106 Amplification of the β -glucosidase (Gene ID: 6212454) from *S. griseus* genomic
107 DNA was performed in a G-Storm GS1 Thermal Cycler (GRI, Promega). Forward,
108 (*bglG_for_NcoI*) 5'CATGCCATGGGGACACACACCCCTGCTTGG3' and
109 reverse (*bglG_rev_EcoRI*) 5'CCGGAATTCTCAGGCTGCCGTGCGCGG3'
110 primers containing *NcoI* and *EcoRI* restriction sites (underlined) respectively were
111 used for the amplification of the β -glucosidase gene. PCR conditions were as
112 follows: denaturation (94 °C for 1 minute), annealing (66 °C for 45 seconds), and
113 extension (72 °C for 1 minute) for 33 cycles with a final extension (72 °C for 5
114 minutes) and an indefinite hold at 4°C. Amplification was carried out with GoTaq®
115 DNA polymerase. Ligation reactions were carried out using T₄ DNA *Ligase* and the
116 resulting recombinant DNA was used for transformation into JM109 *E. coli* cells.

117 The β -glucosidase from *Streptomyces griseus* (*bglG*) was cloned into a *Glutathione-*
118 *S-Transferase* plasmid expression vector (pGEX-4T1).

119

120 **Expression of *S. griseus* β -glucosidase:**

121 *E. coli* transformants were incubated at 37 °C and 220 rpm until an optical density
122 (OD_{600nm}) of 0.4-0.6 was reached. IPTG was added to a final concentration of 0.3
123 mM. The culture was further incubated at 30°C for 6 hours and then centrifuged at
124 5,000 xg for 10 minutes at 4°C to pellet cells. The pellet was washed in 10 mM
125 Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 2.7 mM KCl, pH 7.3. The supernatant
126 was discarded and the pellet was resuspended in 100 ml of cell lysis buffer (10 mM
127 Na₂HPO₄, 1.8 mM KH₂PO₄, 140 mM NaCl, 1% (v/v), Triton X-100, 1 mM DTT,
128 1mM PMSF, pH 7.3). 10 ml of lysozyme (10 mg ml⁻¹) was added to the resuspended
129 cells. Cell lysis was achieved by three cycles of freeze (-196°C, liquid nitrogen) and
130 thaw (at 30°C in a temperature controlled water bath). The lysed cells were
131 centrifuged at 14,000xg for 60 minutes at 4°C. The supernatant containing
132 recombinant protein was syringe filtered (pore size of 0.45 μ m) and used for protein
133 purification.

134

135 **Purification of *S. griseus* β -glucosidase:**

136 Lysed and clarified bacterial supernatant was loaded onto a Glutathione Sepharose
137 4B resin column at a flow rate of 0.5 ml per minute to optimise binding. A specific
138 *PreScission* protease was used for cleavage of fusion protein from the GST tag to
139 yield a highly purified β -glucosidase. This protease cleaved the fusion protein at an
140 rTEV Protease Cleavage Site. The column was washed with 20 ml of cleavage buffer
141 to ensure all cleaved protein was eluted. The eluted enzyme was dialysed against
142 1.0L of 50 mM potassium phosphate buffer, pH 7.2 at 4°C for 36 hours with three
143 changes and constant gentle mixing. Purified protein was concentrated using
144 Centricon® 10 filters (5000xg, 20 minutes, 4°C) with 10 kDa Molecular Weight
145 Cut-Off.

146

147 **Standard assay of β -glucosidase:**

148 β -glucosidase activity was determined spectrophotometrically at 37°C using the
149 substrate *p*-nitrophenyl- β -D glucopyranoside (pNPG). Briefly, 20 μ l of appropriately
150 diluted enzyme (typically 0.5 μ g μ l⁻¹), was mixed with 120 μ l of 50 mM potassium

151 phosphate buffer, pH 7.2. The assay was initiated with 30 μ l of 7.0 mM pNPG (in 50
152 mM potassium phosphate buffer, pH 7.2) After 20 min the reaction was stopped by
153 adding 30 μ l of 1M Na₂CO₃ solution The mustard yellow colour, developed due to
154 the liberation of *p*-nitrophenol, was monitored at 405nm in a microplate reader
155 (BioTek PowerWave). For immobilised enzyme the reaction mixture was centrifuged
156 to remove nanoparticles before measurement.

157

158 **Effect of pH and temperature on *S. griseus* β -glucosidases:**

159 The pH optimum for *S. griseus* β -glucosidases activity was determined in three
160 different buffers: pH 3.5-5.5: 50 mM sodium citrate, pH 6.0-8.0: 50 mM potassium
161 phosphate and pH 8.5-9.0: 50 mM glycine. The temperature optimum for *S. griseus*
162 β -glucosidase was obtained from measurements of activity (at the optimum pH of
163 6.9) in the temperature range from 20 °C to 100 °C. Thermostability was assessed
164 by incubating an enzyme preparation (free or immobilised) at the appropriate
165 temperature for 3 hours: samples were withdrawn at intervals of 20 minutes and
166 tested using the standard assay. All assays were subject to three intra- and inter-
167 experimental repeats.

168

169 **Influence of effectors on *S. griseus* β -glucosidase:**

170 All effectors investigated were used at a final concentration of 1 mM. The final
171 concentration of solvent used was 40% (v/v) in all cases. Inhibition of *S. griseus* β -
172 glucosidase by glucose was carried out using different concentrations (ranging 0.056
173 to 0.336 mM) of glucose.

174

175

176 **Kinetics constants for *S. griseus* β -glucosidase:**

177 Kinetic parameters were calculated from an activity *versus* substrate concentration
178 plot based on triplicate independent assay results by regression analysis using the
179 software EnzFitter v2.o.18.0 (Biosoft, Cambridge, UK). Kinetic parameters were
180 obtained using the substrate *p*-nitrophenyl- β -D glucopyranoside (pNPG) using a
181 range of substrate concentrations from 4 to 30 mM.

182

183 **Nanoparticle immobilisation:**

184 The immobilisation of β -glucosidases onto zinc oxide (ZnO) nanoparticles (NPs) was
185 carried out by incubating 1 ml of enzyme ($50 \mu\text{g ml}^{-1}$) with 1.0 ml of nanoparticles
186 (1.0 mg/ml) with gentle mixing. The immobilisation was carried out for 90 minutes
187 at 4°C.

188

189 **Synthesis of glucosides:**

190 Synthesis of hexyl- β -D-glucoside was performed in a total reaction volume of 1 ml.
191 A 160 μl aliquot of a solution containing Glucose (10 mM) and β -glucosidase (0.1-
192 1.0 mg/ml) in 50 mM potassium phosphate buffer pH 6.9 was incubated with 50 μl
193 acetonitrile for 1 hour. An alcohol substrate (790 μl) was then added to initiate the
194 reverse hydrolysis reaction. The reaction was allowed to proceed at 60°C with
195 agitation on a shaking incubator (Innova, 200 rpm) for a further hour. A sample of
196 supernatant containing the solvent phase was withdrawn for TLC analysis.

197

198 **Thin Layer Chromatography (TLC) of glucosides:**

199 Enzymatically synthesised glucosides were separated on thin layer chromatography
200 (TLC) plates using silica gel 60 F254nm aluminium sheets (dimension: 20 X 20 cm).
201 Briefly, 2.0 μl of each reaction product was applied to a TLC plate by capillary
202 injection with a disposable micropipette. Spotted samples were fixed and activated at
203 110 °C for 30 minutes. Plates were developed in a mobile phase of 1-propanol/ethyl
204 acetate/water (6:2:2,v/v/v). The plates were dried at room temperature for 5 minutes
205 and sprayed using an atomizer (TLC sprayer) containing 0.3% (w/v) N-(1-naphthyl)
206 ethylenediamine dihydrochloride and 5% (v/v) H₂SO₄ in methanol. The plates were
207 oven dried at 110°C until coloured spots were observed.

208

209

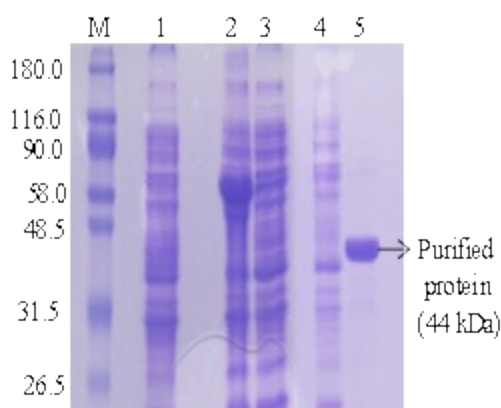
210 **Error Estimates**

211 Unless indicated, replicate errors were within the area of the data points drawn on the
212 graphs shown below.

213 **Results and Discussion**

214 **Expression and Purification of *S. griseus* β -glucosidase**

The molecular weight (44 kDa) of purified *S. griseus* β -glucosidase and its pI (4.87) were established using Expasy tools (<http://web.expasy.org/protparam>). A highly purified preparation of *S. griseus* β -glucosidase was readily prepared using the protocols described herein (Figure 1).



215 **Figure 1: SDS-PAGE of *S. griseus* β -glucosidase purification.** SDS gel
216 electrophoresis of purification stages for *S. griseus* β -glucosidase: Lane M is a broad
217 range protein markers (kDa), Lane 1: Non-induced cell lysate, Lane 2: IPTG induced
218 cell lysate, Lane 3: Glutathione Sepharose 4B column flow through, Lane 4: Column
219 washing: Lane 5: Eluted purified β -glucosidase.

220

221 This report is the first on the expression and purification of this novel enzyme.
222 Initially the expression proved problematic due to the formation of insoluble protein.
223 This problem was solved when the *Glutathione-S-Transferase* based expression
224 vector pGEX-4T1 was chosen for cloning and the enzyme was overexpressed as a
225 fusion protein with GST. The GST tag was proteolytically removed before
226 characterisation of the purified enzyme. The specific activity of purified protein
227 ($217 \pm 5.0 \mu\text{mol}/\text{min}/\text{mg}$) was roughly 100 times higher than the crude extract (2.15
228 $\mu\text{mol}/\text{min}/\text{mg}$) of β -glucosidase. A typical purification starting with 500 ml of
229 culture yielded 3 to 4 mg of purified protein.

230

231 **Effect of pH and temperature on *S. griseus* β -glucosidase**

232 The effect of pH and temperature on the activity of *S. griseus* β -glucosidase was
233 examined (Figure 2. A,B). The commercially available β -glucosidase from sweet
234 almond was used as a comparator for these studies since it is a well characterised
235 enzyme widely used for a variety of biotechnological applications.

236

237 *S. griseus* β -glucosidase had a pH optimum of 6.9 (Figure 2A), slightly higher than
238 commercial almond β -glucosidase (pH 6.6) under the same assay conditions. β -
239 glucosidases typically have pH optima in the region of 3 to 7 ([15]; [16]; [17]; [18];
240 [19]) and the *S. griseus* enzyme lies at the upper end of this range. Activity at higher
241 pH values may be useful for specific industrial applications where processing is
242 carried out under more alkaline conditions *e.g.* the paper pulp industry ([20]; [21]).

243

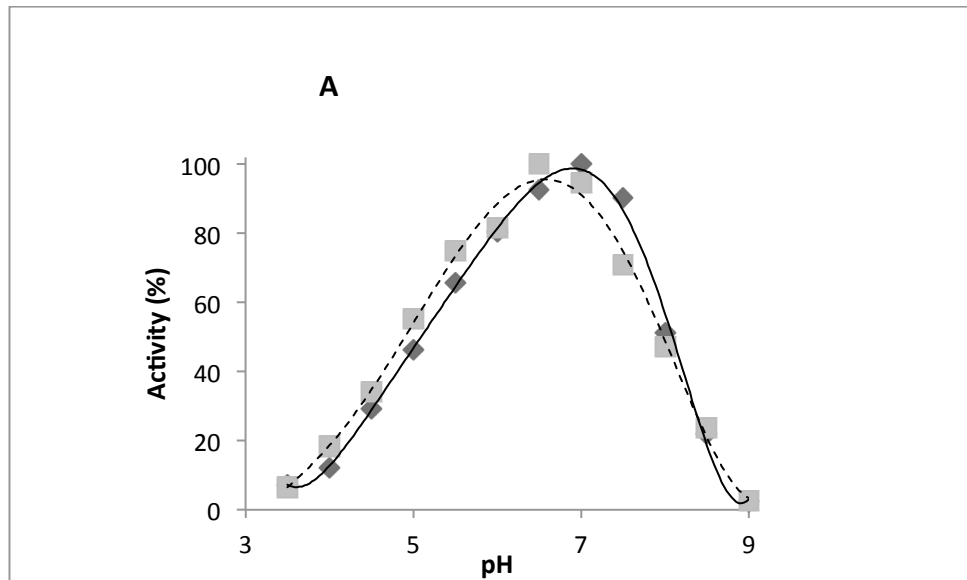
244 The effect of temperature on activity was examined in the range 20 °C to 100 °C.
245 The optimum temperature of *S. griseus* β -glucosidase was estimated to be 69 °C.
246 Under the same conditions the commercially available almond β -glucosidase showed
247 a temperature optimum of 59 °C. The profile for *S. griseus* β -glucosidase
248 immobilised on a nanoparticle support is also shown (Figure 2.B): immobilisation
249 increased the enzyme's optimum temperature to 75 °C. This probably reflects an
250 increase in stability at elevated temperatures that is often seen with immobilised
251 enzymes.

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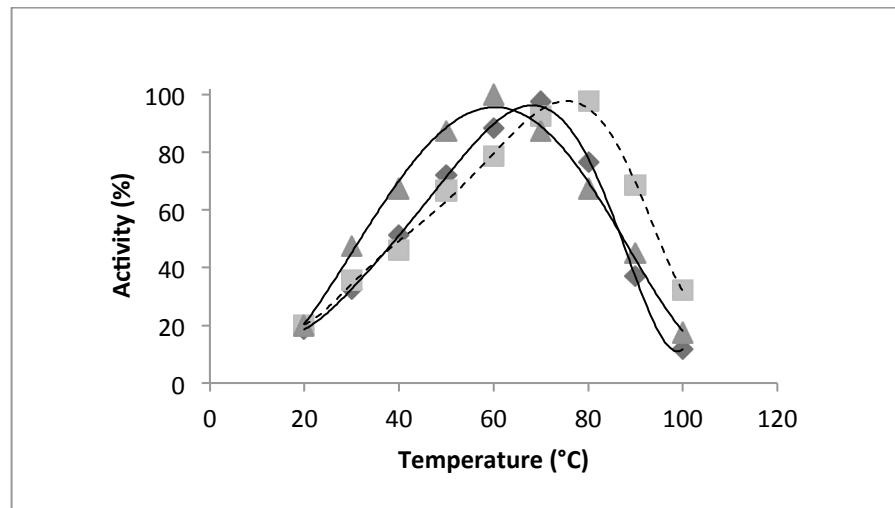


256

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259 **B**



260

261

262

263 **Figure 2: Effect of pH and temperature on the activity of recombinant *S. griseus***

264 **β -glucosidase – comparison with commercial Almond enzyme. A. Enzyme**

265 activity was estimated in the pH range from 3.5 to 9.0. *S. griseus* β -glucosidase (♦);

266 commercial Almond β -glucosidase (■). B. Optimum temperature was estimated over

267 the range 20 °C to 100 °C. *S. griseus* β -glucosidase (♦); commercial Almond β -

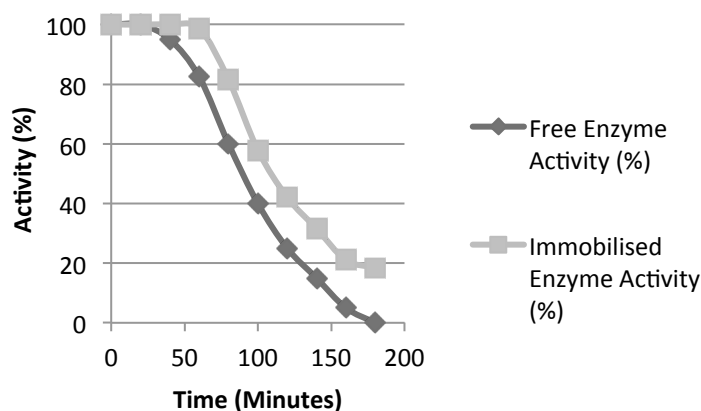
268 glucosidase (▲) nanoparticle immobilised *S. griseus* β -glucosidase (■).

269

270 A recent review of glucosidases and their industrial applications, [20], listed only one
271 enzyme, the hyperthermostable glucosidase from *Thermus thermophilus* HJ6, with a
272 pH optimum higher than 7.0 and a temperature optimum higher than 70 °C. The *T.*
273 *thermophilus* enzyme had a pH optimum of 8.5 and a temperature optimum of 90 °C
274 ([22]). The *S. griseus* enzyme, therefore, is close to the upper end of the range for
275 most glucosidases examined to date in terms of both pH optimum and temperature
276 optimum.

277 Thermostability of the *S. griseus* enzyme was examined by incubating the enzyme at
278 its optimum temperature (69 °C) and monitoring activity over time (Figure 3). This
279 figure also shows how thermostability is increased when the enzyme is immobilised
280 on a nanoparticle support.

281



282

283 **Figure 3: Thermal stability of free and immobilised *S. griseus* β -glucosidase.**

284 Immobilisation and thermal stability monitoring as described in Materials and
285 Methods. Reactions were followed for a three hour period using the standard assay.

286 *S. griseus* β -glucosidase (\blacklozenge); nanoparticle immobilised *S. griseus* β -glucosidase (\blacksquare).

287

288 The free enzyme retained more than 50% of its activity after incubation at 69°C for
289 1.5 hours while the immobilized form retained more than 50% activity for roughly
290 two hours under the same conditions. After three hours, the free enzyme became
291 inactivated while the immobilised form still retained *ca* 20% activity. In terms of
292 thermostability, the *S. griseus* enzyme is comparable to β -glucosidases commonly
293 used for biocatalytic applications ([17]; [18];[19]). Thus, it is somewhat better than
294 the thermostable β -glucosidase from *Penicillium citrinum* that has an optimum

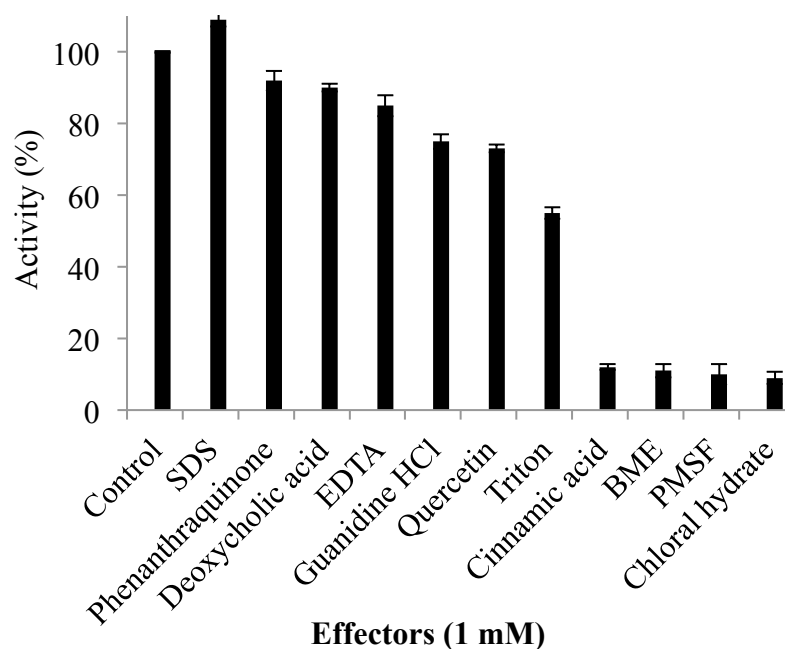
295 temperature of 70 °C but becomes inactivated above 60 °C ([23]) or that from
296 *Aspergillus fumigatus* Z5 ([17]) which had maximal activity at 60 °C but lost activity
297 rapidly above this temperature. On the other hand, some highly thermostable
298 enzymes have been reported: the glucosidase recently described from *H. orenii*
299 retained more than 90 % of its activity after 3 h of incubation at 65 °C ([24]) while
300 that from *Dictyoglomus thermophilum* reportedly retained a remarkable 70–80% of
301 its initial activity after 7 days of incubation at 70 °C ([25]).

302

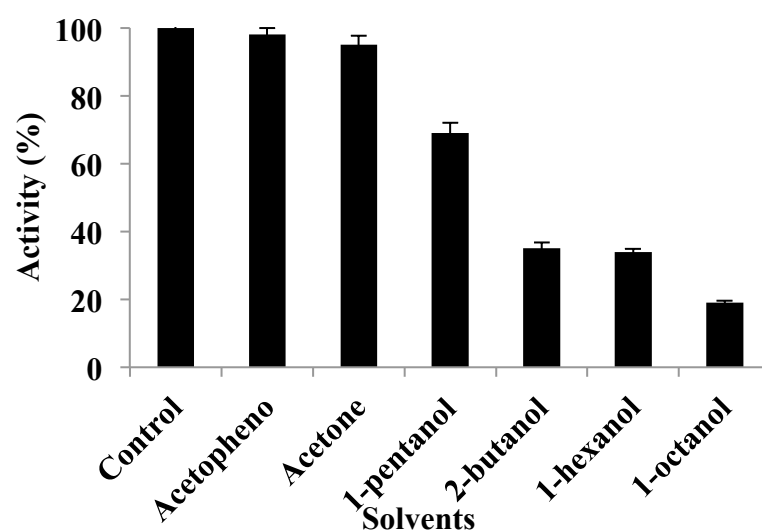
303 **Effectors of *S. griseus* β -glucosidase**

304 The activity of β -glucosidase was examined in presence of a final concentration of
305 1.0 mM of various effectors: SDS, phenanthraquinone, deoxycholic acid, EDTA,
306 guanidine hydrochloride, quercetin, Triton X-100, cinnamic acid, BME, PMSF, and
307 chloral hydrate (Figure 4A).

308



309



310

311

312 **Figure 4: The influence of effectors and solvents on *S. griseus* β -glucosidase**

313 **activity.** Assays were carried out in the absence (control) and presence of 1.0 mM

314 effectors at 37°C. **A** Effectors of *S. griseus* β -glucosidase activity: SDS is sodium

315 dodecyl sulphate; EDTA is Ethylene diaminetetraacetic acid; BME is β -

316 Mercaptoethanol and PMSF is phenyl methane sulfonyl fluoride. Activity was

317 expressed as a percentage of a control reaction without effectors. **B** Effect of solvents

318 on *S. griseus* β -glucosidase activity. Assays were carried out in the absence (control)

319 and presence of 40% (v/v) solvents at 37°C.

320

321 Inhibition by the thiol modifying compounds, β -mercaptoethanol and PMSF,

322 suggests the involvement of thiols in the activity of this protein. The enzyme was

323 slightly activated by SDS but inhibited by Triton. The inhibition with Triton might

324 be attributed to its aromatic moiety interacting with a hydrophobic region close to or

325 in the active site. Cinnamic acid has an aromatic ring that may inhibit in a similar

326 manner. Inhibition by chloral hydrate was surprising since it is a rather small

327 molecule. It is a fully hydrated aldehyde which is present in solution as a gem-diol

328 structure and it is possible that this diol is interacting at the glucose binding site.

329

330 Solvent tolerance is a highly desirable trait for enzymes that are to be used for

331 industrial chemoenzymatic synthesis [20]: it allows the application of such enzymes

332 in a wider range of solvent environments and by reducing water activity increases the

333 yield of reverse hydrolysis reactions. The stability of *S. griseus* β -glucosidase was

334 examined in the presence of organic solvents (final concentration of 40% (v/v)). The
335 solvents used were: acetophenone, acetone, 1-pentanol, 2-butanol, 1-hexanol, and 1-
336 octanol. It was found that 1-octanol, 1-hexanol, and 2-butanol inhibited enzyme
337 activity by 19%, 34%, and 35%, respectively (Figure 4B). Thus, the primary alcohol
338 substrates were shown to inhibit the enzyme and this inhibition increased with chain
339 length of the alcohol. Significantly, this inhibition did not impair alkyl glucoside
340 synthesis (see below). The increasing inhibition seen with increasing chain length
341 suggests that alcohols are binding to a hydrophobic region that inhibits substrate
342 turnover but does not inactivate the enzyme.

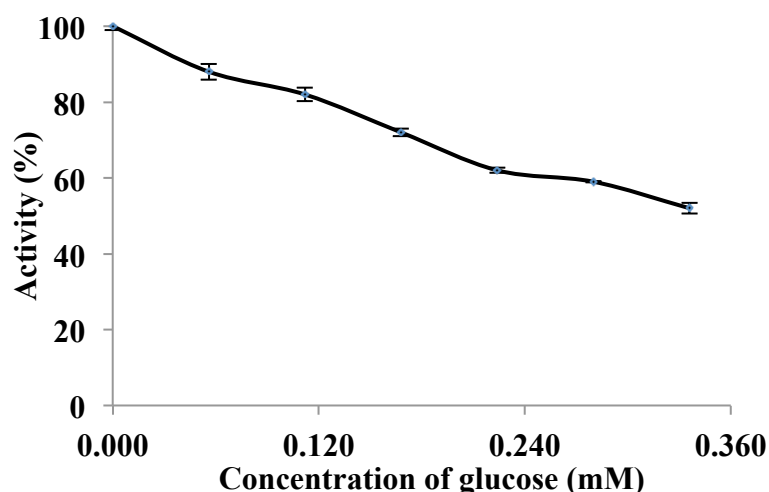
343

344 **Inhibition by glucose:**

345 Applications where glucosidases are used to hydrolyse glycosidic bonds require an
346 enzyme that is insensitive to product inhibition. Different concentrations of glucose;
347 ranging from final concentrations of 0.056-0.336 mM were tested (Figure 5). A
348 glucose concentration of 0.336M reduced activity by 50%.

349

350



351

352 **Figure 5: Inhibition of *S. griseus* β -glucosidase by glucose.** Inhibition studies
353 were carried out at 37 °C in 50 mM potassium phosphate buffer, pH 6.9 using the
354 standard assay. Different concentrations of glucose were used in the ranges from
355 0.056-0.336 mM. The percentage activity of control reaction was measured without
356 glucose.

357

358 A concentration of 0.336 mM glucose caused 50% loss of activity in the standard
359 assay. In general, most microbial β -glucosidases are inhibited in the presence of
360 glucose ([26]; [27]; [28]). However, a few glucose tolerant enzymes have been
361 reported such as those of *Candida peltata* and *Aspergillus oryzae* ([29]; [30]). On the
362 other hand, the fungal β -glucosidase from *Aspergillus niger* lost 85% activity in the
363 presence of 0.168 mM glucose ([31]). This tight binding of glucose observed in the
364 present work rules out applications where hydrolysis is employed such as bioethanol
365 production. However, tight binding of this substrate is desirable for chemoenzymatic
366 synthetic applications involving reverse hydrolysis.

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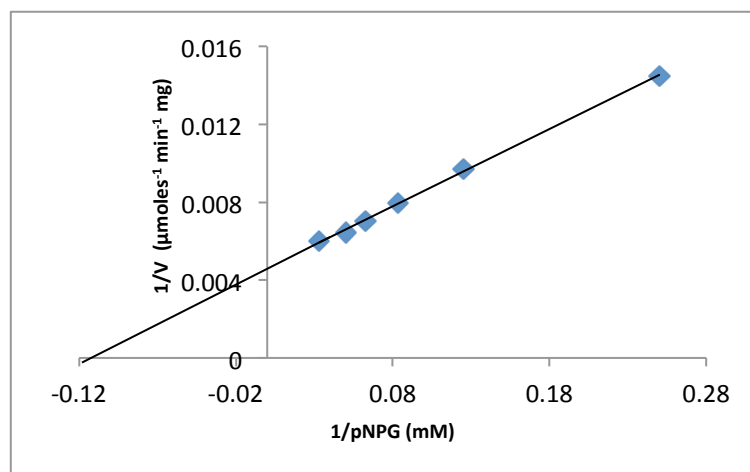
369

370 **Kinetic constants**

371 Initial rates of β -glucosidase were determined using different concentrations of *p*-
372 nitrophenyl- β -D glucopyranoside (pNPG) between 4-30 mM. The value K_m and V_{max}
373 were obtained as 8.6 ± 0.5 mM and $217 \pm 5.0 \mu\text{moles}^{-1} \text{min}^{-1} \text{mg}$, respectively (Figure 6).

374

375



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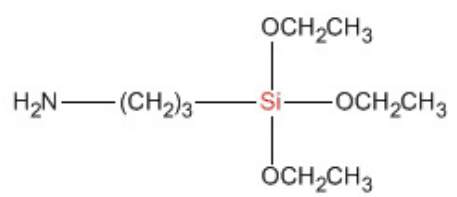
378 **Figure 6. Kinetic constants estimation for hydrolysis of *p*-nitrophenyl- β -D**
379 **glucopyranoside (pNPG) by *S. griseus* β -glucosidase.** Assays were performed at 37
380 °C using the standard assay while varying the concentration of pNPG.

381

382 **Nanoparticle immobilisation:**

383 The findings above indicate that immobilisation on a nanoparticle support has a
384 significant effect on thermostability of the *S. griseus* enzyme. Immobilisation
385 typically enhances an enzyme's thermal stability as well as improving its solvent
386 tolerance. In this study, Zinc oxide (ZnO) nanoparticles modified with γ -
387 aminopropyltriethoxysilane (KH550) were chosen based on their cost effectiveness.
388 KH550 is an amino-functional coupling agent which provides hydrophobicity to the
389 surface of ZnO particles (Scheme 1).

390



391

392

393 **Scheme1: Structure of γ -aminopropyltriethoxysilane (KH550).** ZnO

394 nanoparticles are coated with KH550 which provides for ease of dispersion of the
395 nanoparticles.

396

397 The *S. griseus* enzyme was found to stick tightly to the Zn nanoparticles used in this
398 study and was not removed even after extensive washing. Nanoparticle immobilised
399 enzyme was washed with buffer solutions containing up to 2.0M KCl without eluting
400 significant enzyme activity. Only 6% of enzyme activity was removed after 10
401 washes with a 2.0M KCl solution. Similarly, washes in up to 50% (v/v) acetonitrile
402 caused no loss of activity.

403

404 The ease of immobilisation is of considerable value since the chemical steps, costs
405 and resulting loss of activity involved in covalent immobilisation are avoided. A
406 similarly facile immobilisation has been reported for a β -galactosidase from
407 *Aspergillus oryzae* [32]. The immobilised enzyme exhibited the same pH optimum as
408 the free enzyme but its optimum temperature was shifted to 75 °C (Figure 2B).
409 Although immobilisation enhanced thermostability, it was not as dramatic an
410 improvement as seen with some other enzymes. This may be due to the method of
411 immobilisation which does not involve multiple covalent points of attachment that
412 are known to stabilise immobilised enzymes. Recently, Verma and coworkers [13]
413 showed a dramatic increase in the stability of *Aspergillus niger* glucosidase following
414 covalent immobilisation on magnetic nanoparticles: the free enzyme lost all activity
415 after 2 h of incubation at 70 °C whereas the immobilized form still retained 67% of
416 its initial activity after 4 h incubation.

417

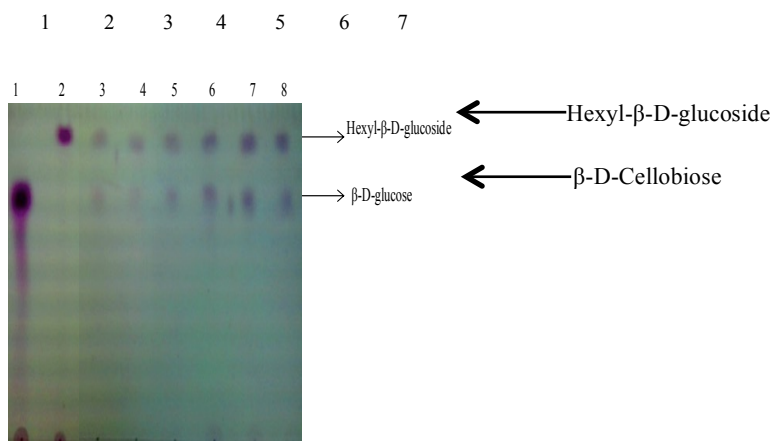
418 **Synthesis of Alkyl glucosides:**

419 The application of the immobilised enzyme to the synthesis of hexyl-glucoside was
420 explored. Such glucosides are important biodegradable surfactants with a wide range

421 of food and pharmaceutical applications [33]. Figure 7 shows a time course of hexyl
422 glucoside synthesis.

423

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428

429 **Figure 7: Synthesis of hexyl glucoside using immobilised *S. griseus* β-**
430 **glucosidase.** Thin layer chromatography of hexyl-β-D-glucoside synthesis. 20 μl of
431 reaction mixture was withdrawn at different times and 4.0 μl of each sample was
432 analysed by TLC: Lane 1, Standard Hexyl-β-D-glucoside; Lane 2, 2 hours of
433 incubation; Lane 3, 4 hours of incubation; Lane 4, 8 hours of incubation; Lane 5, 16
434 hours of incubation; Lane 6, 24 hours of incubation and lane 7, 30 hours of
435 incubation.

436

437 In addition to hexanol this enzyme was able to utilise a wide range of alcohol
438 substrates as acceptor. Thus, propyl, butyl, pentyl, octyl, benzyl and 2-phenyl-ethyl
439 glucosides were also synthesised by this using the same conditions by simply
440 replacing the alcohol substrate (data not shown). These alcohol substrates were
441 shown to inhibit the glucosidase activity of the enzyme (see above) but clearly do not
442 inhibit the synthesis of alkyl glucosides. This breadth of substrate specificity is a
443 significant advantage for the application of this enzyme. The glycosidation of
444 bioactive compounds, for example, has been pursued to enhance their stability,
445 solubility or receptor binding properties ([7]). Our initial studies establish that this

446 reaction proceeds readily for this enzyme but further work is clearly required to
447 define the full extent of this synthetic capability.

448

449 **Conclusions:**

450 The β -glucosidase from *S. griseus* has a number of characteristics that make it
451 suitable as a potential novel biocatalyst for industrial applications: it is optimally
452 active at neutral pH, thermostable up to 69 °C, solvent tolerant and easily
453 immobilised on a nanoparticle support. It has been shown to readily catalyse alkyl
454 glucoside synthesis. The characteristic features of this recombinant *S. griseus* β -
455 glucosidase suggest that it may offer a useful alternative to currently used β -
456 glucosidases for certain green chemistry applications.

457

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