

1 Plant cellulose synthase membrane protein isolation
2 directly from *Pichia pastoris* protoplasts, liposome
3 reconstitution, and its enzymatic characterization

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8 **Abstract**

9 The most abundant renewable biopolymer on earth, viz., cellulose, acts as carbon storage reserve
10 in plant and microbial cell walls that could potentially be converted into biofuels or other
11 valuable bioproducts. Cellulose is synthesized by a plant cell membrane-integrated processive
12 glycosyltransferase (GT) called cellulose synthase (CesA). Since only a few of these plant CesAs
13 have been purified and characterized to date, there are huge gaps in our mechanistic
14 understanding of these enzymes. Furthermore, the coordination between different CesAs
15 involved in primary and secondary cell wall formation is yet to be unveiled. The biochemistry
16 and structural biology studies of CesAs are currently hampered by challenges associated with
17 their expression and extraction at high yields. To aid in understanding CesA reaction

18 mechanisms and to provide a more efficient CesA extraction method, two putative plant CesAs –
19 PpCesA5 from *Physcomitrella patens* and PttCesA8 from *Populus tremula x tremuloides* that
20 are involved in primary and secondary cell wall formation in plants were expressed using *Pichia*
21 *pastoris* as an expression host. We developed a protoplast-based membrane protein extraction
22 approach to directly isolate both these membrane-bound enzymes for purification, as detected by
23 immunoblotting and mass spectrometry-based analyses. Our method results in a higher purified
24 protein yield by 3-4-fold than the standard cell homogenization protocol. Our purified CesAs
25 were reconstituted into liposomes to yield active enzymes that gave similar biochemical
26 characteristics (e.g., substrate utilization and cofactor requirements, no primer needed to initiate
27 polymerization reaction) as enzymes isolated using the standard protocol. This method resulted
28 in reconstituted CesA5 and CesA8 with similar Michaelis-Menten kinetic constants, $K_m = 167$
29 μM , $108 \mu\text{M}$ and $V_{\max} = 7.88 \times 10^{-5} \mu\text{mol}/\text{min}$, $4.31 \times 10^{-5} \mu\text{mol}/\text{min}$, respectively, in concurrence
30 with the previous studies. Taken together, these results suggest that CesAs involved in primary
31 and secondary cell wall formation can be expressed and purified using a simple and more
32 efficient extraction method. This could potentially help unravel the mechanism of native and
33 engineered cellulose synthase complexes involved in plant cell wall biosynthesis.

34 **Keywords**

35 Cellulose, Cellulose synthases, Membrane proteins, *Pichia pastoris*, Protoplasts, UDP-Glucose

36

37 **1. Introduction**

38 Polysaccharides are a major class of natural polymers found in the plant, animal, and microbial
39 kingdoms that are essential in providing energy, structural support, and other biological functions

40 [1–3]. These complex carbohydrates are synthesized by a group of enzymes called
41 polysaccharide synthases [4–7]. Some of these polysaccharide synthases are membrane-
42 integrated processive family-2 glycosyltransferases, such as cellulose, hyaluronan, chitin, and
43 alginate synthases [8]. Polysaccharides such as cellulose and hemicellulose are the most
44 abundant renewable polymers found in plant cell walls. Cellulose, an unbranched
45 homopolysaccharide made up of D-glucose linked by β -1,4-glycosidic bonds [9], is the major
46 structural component of plant cell walls and is also found in algae and some microbes. It is used
47 in several industries, including but not limited to paper, textiles, and furniture. In recent decades,
48 cellulose and its associated proteins have gained much attention since it could be used as a
49 potential feedstock for producing bioethanol and other valuable bioproducts [10–13]. Therefore,
50 increasing plants' biomass yield and sugar content is imperative and possible by altering the cell
51 wall composition [14]. However, understanding the fundamental mechanisms and factors
52 influencing the formation of these polysaccharides is far from fruition.

53 Cellulose is processively synthesized by a membrane-integrated processive family-2
54 glycosyltransferase called cellulose synthase (CesA). These enzymes exist in nature as
55 membrane-localized complexes [15–17] and typically contain multiple monomers that
56 coordinate amongst themselves and carry out various biological functions. For instance, in
57 *Arabidopsis thaliana*, CesAs interact to form rosette subunits, and six of these subunits assemble
58 into multimeric rosette complexes, often called cellulose synthase complexes (CSCs). These
59 CSCs contain several different CesA isoforms that express differentially during various stages of
60 cell wall formation [18,19]. *Arabidopsis* expresses ten different *CesA* genes with different
61 subsets that are involved in either primary cell wall formation (proteins encoded by *CesA1*,
62 *CesA3*, and *CesA6* or *CesA2/5/9*) or secondary cell wall formation (proteins encoded by *CesA4*,

63 *CesA7*, and *CesA8*) [20]. Recently, the structure of a homotrimeric CSC containing three *CesA8*
64 monomers from Poplar was solved using CryoEM, which revealed a molecular basis for
65 understanding cellulose microfibril formation [17]. Each *CesA* monomer comprises seven
66 transmembrane helices circumscribed by the intracellular N- and extracellular C-terminus and a
67 large cytosolic GT domain. Likewise, the homotrimeric structure of *CesA7* from cotton was also
68 resolved in a similar manner and showed an analogous structure [21].

69 Plasma membrane-localized CSCs are made up of different individual *CesA* isoforms that are
70 responsible for processively synthesizing single glucan chains and assembling them into the
71 cellulose microfibril (CMF) matrix. Recent biochemical studies show that a single *CesA* isoform,
72 when functionally reconstituted into a liposome, is enough to synthesize cellulose microfibrils or
73 form UDP as a bi-product when incubated with UDP-glucose as substrate [22–24].

74 Although seminal research in the last couple of years has revealed the structure of plant *CesA*
75 and its activity *in vitro*, there are still significant gaps in our understanding of how these *CesA*
76 monomers coordinate together and form microfibrils both *in vivo* and *in vitro*. It is important to
77 note that such an imperative plant protein system has only a few reports available on their
78 expression and purification to date. This is mainly due to the lack of reports that elucidate
79 simple, efficient, and feasible methods of expression and purification. In this work, we intend to
80 showcase an efficient method of purification that could potentially help prepare and study
81 different *CesAs* side by side. To achieve this, we selected two putative *CesAs* (*CesA5* from
82 *Physcomitrella patens* and *CesA8* from *Populus tremula x tremuloides*) involved in the primary
83 and secondary cell wall formation. Both these enzymes were expressed heterologously in *Pichia*
84 *pastoris* and purified using a modified protoplast extraction method, as confirmed by various
85 detection methods. The enzymes were reconstituted into proteoliposomes and produced UDP

86 when incubated with UDP-Glucose as a substrate and manganese as a cofactor. We have
87 performed steady-state kinetic analysis and determined various kinetic parameters for both the
88 enzymes. To our knowledge, this is the first study in which enzymes involved in both primary
89 and secondary plant cell wall formation have been studied side by side *in vitro*. Overall, our
90 results show that the modified extraction approach is suitable for both Cesa5 and Cesa8 without
91 impacting the catalytic activity.

92 **2. Materials and methods**

93 ***2.1 Cloning and transformation into yeast***

94 Cellulose synthase 8 (*CesA8*) gene from hybrid aspen (*Populus tremula x tremuloides*) carrying a
95 C-terminal dodeca-HIS-tag and an N-terminal FLAG tag [17], and Cellulose synthase 5 (*CesA5*)
96 gene from moss (*Physcomitrella patens*) carrying a C-terminal dodeca-HIS-tag [23] was custom
97 synthesized from GenScript and cloned into yeast expression vector pPICZA. Plasmid maps for
98 both constructs are shown in Figure S1. Protein sequences for Cesa5 and Cesa8 are shown in
99 Supplementary text S1. The construct was then transformed into the *Pichia pastoris* SMD1168H
100 strain (single protease deficient strain) using the Easysselect Pichia Expression kit (Invitrogen,
101 Cat# K174001) according to the manufacturer's specifications. The cells were plated on YPDS
102 plates [1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar (w/v)] containing 100
103 µg/mL zeocin and were incubated for 2-4 days at 30 °C. The colonies were screened using
104 colony PCR by checking the integration of the gene into alcohol oxidase (AOX I) loci using
105 primers specific to the AOX promoter (5' AOX 1 primer: 5'-
106 GACTGGTTCCAATTGACAAGC-3' and 3' AOX 1 primer - 5'-

107 GCAAATGGCATTCTGACATCC-3'). All the primers used in this study were obtained from
108 Integrated DNA Technologies and are tabulated in Supplementary Table S1.

109 ***2.2 Growth conditions for expression of CesaA proteins***

110 Growth conditions were similar to the one mentioned in Purushotham et al., with slight
111 modifications [22]. Transformed cells were cultured overnight at 30°C in 5 ml YPDS culture
112 tubes. Approximately 3-5% of this culture was inoculated into 300 ml preculture media (BMGY
113 medium containing 100 mM Phosphate buffer pH 6.0, 1% yeast extract, 2% peptone, 1.34%
114 yeast nitrogen base, 1% glycerol) and incubated overnight at 30°C and 300 rpm. Cells were
115 collected after 12-16 hours and resuspended to an OD₆₀₀ of 0.4 in BMMY induction media
116 (BMGY medium supplemented with 0.5% methanol instead of glycerol). Induction was carried
117 out in baffled flasks at 20°C and 300 rpm for 24 h. The cells were later harvested at 7000 rpm for
118 20 mins, and the cell pellets were directly used for purification or stored at -80°C for long-term
119 storage.

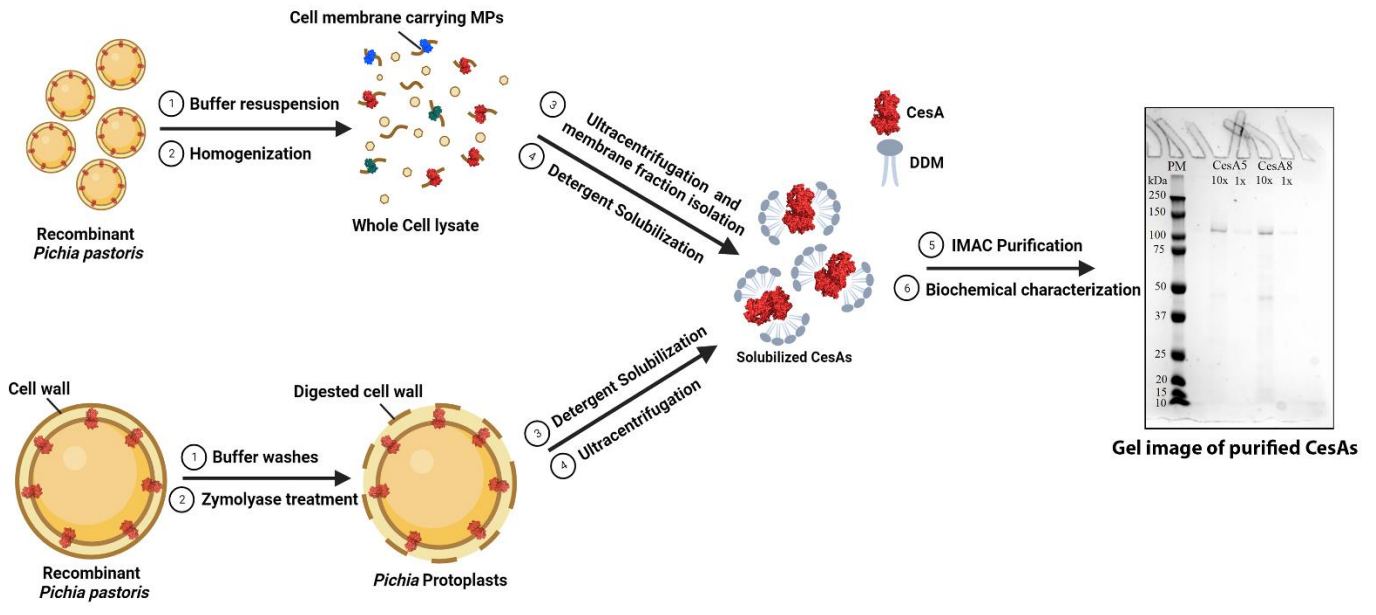
120 ***2.3 Extraction and purification of enzymes using Pichia protoplasts***

121 Traditional methods of protein extraction from yeast, such as sonication, bead-beating, and
122 homogenization, were employed to move the membrane protein (MP) from the cell surface to the
123 soluble fraction. However, the sonication or the bead beating methods were futile since the both
124 of them resulted in no yield at all. For homogenization method, we resuspended 12g of harvested
125 cells from 1L culture in 60 mL lysis buffer (20 mM Tris-HCl pH 7.5, 0.6 M sorbitol) and lysed
126 by two passes through a homogenizer at ~15,000 psi in the presence of one cComplete™ EDTA-
127 free protease inhibitor tablet per 50 mL sample volume. The lysate was centrifuged at 19,000 × g
128 for 10 min at 4 °C, and the supernatant was centrifuged for 2 h at 100,000 × g and 4 °C to pellet

129 the membrane fraction. The membrane pellet was solubilized in 60 mL membrane resuspension
130 buffer (MRB) (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 40 mM n-Dodecyl β -D-Maltoside
131 (DDM), 10% vol/vol glycerol, and one cOmplete™ EDTA-free protease inhibitor tablet) and
132 incubated at 4 °C for 120 mins with gentle agitation. Insoluble material was removed by
133 centrifugation at $100,000 \times g$ for 30 min at 4 °C. Supernatant was then subjected to IMAC based
134 purification as mentioned in the next section. However, the high-pressure homogenizer method
135 resulted in a very low yield of CesA proteins (Fig. S2).

136 Hence, we used a gentler method involving a short solubilization step directly on whole cells to
137 favor the extraction of the undamaged, correctly folded MPs targeted to the plasma membrane
138 [25]. In this method, 12g of harvested cells from 1L culture was initially washed with 200 mL
139 double-distilled water to remove any residual media, followed by 200 mL SED buffer (1M
140 Sorbitol, 25 mM EDTA, and 1M DTT). The cells were later washed using 200 mL of 1M
141 sorbitol before resuspending them in 150 mL of CG buffer (20 mM trisodium citrate pH 5.8,
142 10% glycerol, 1 mM PMSF). 20 units of zymolyase (from Amsbio, UK) per gram of cells was
143 added to the mixture and incubated at 30°C and 70 rpm for 20-30 mins. The resulting yeast
144 spheroplasts or protoplasts (yeast cells without a cell wall) were later used to directly solubilize
145 membrane proteins in 100 mL solubilization buffer (containing 50 mM Tris-HCl pH 7.4, 500
146 mM NaCl, 10% glycerol, 20 mM imidazole, 40 mM DDM, and cOmplete™ EDTA-free protease
147 inhibitor tablet). After solubilizing the membrane proteins for 2-2.5 h at 4°C with gentle
148 agitation, the samples were centrifuged at $48,400 \times g$ at 4°C for 1 h in a Beckman Coulter JA-20
149 fixed angle rotor. The supernatant was collected and filtered using a 0.22 μ m non-sterile syringe
150 filter and incubated with 5 ml preequilibrated TALON superflow resin (Cytiva, Cat# 28957502)

151 overnight at 4°C with gentle agitation. The schematic workflow of the traditional and protoplast-
152 based lysis methods is shown in Fig. 1.



154 **Figure 1.** Schematic workflow of CesA purification methods. (*Top*) Recombinant *Pichia* cells
155 expressing the CesA (shown in red) lysed using the homogenization method followed by
156 membrane fraction extraction, detergent solubilization using N-dodecyl β -D-maltoside (DDM),
157 and purification. (*Bottom*) Protoplasts-based extract method through multiple buffer washes
158 (double-distilled water, SED, Sorbitol) and Zymolyase treatment. Zymolyase digests cell walls,
159 forming protoplasts that were then directly used for protein solubilization using DDM. The gel
160 on the right is a Coomassie-stained SDS PAGE gel depicting 1x and 10x concentrated CesA5
161 and CesA8. Proteins depicted in red are CesA8 (PDB:6WLB). MP: Membrane proteins. The gel
162 image is not to scale.

163

164

165

166 **2.4 Gravity-based IMAC purification of CesaA5 and CesaA8**

167 The resin was packed into a gravity flow column and sequentially washed with equilibration
168 (EQ) buffer (20 mM Tris-HCl pH 7.5, 100 mM NaCl, 10% glycerol) containing 20-, 40-, or 60-
169 mM imidazole and 1 mM LysoFoscholineEther-14. For CesaA5, an additional washing step with
170 EQ buffer containing 80 mM imidazole and 1 mM LysoFoscholineEther-14 was required before
171 the final elution. CesaA proteins were eluted in the EQ buffer containing 300 mM imidazole. The
172 eluted fraction was concentrated 10 times using 100 kDa Amicon Ultra-15 centrifugal filters
173 (Millipore Sigma, Cat# UFC903008) and buffer exchanged onto a pre-equilibrated PD-10
174 column (Cytiva, Cat# 17085101). The concentration of the purified protein was estimated using
175 BCA assay (ThermoFisher, Cat# 23225). Samples were stored at 4°C and reconstituted
176 immediately. For long-term storage, it is recommended to flash freeze the samples and store
177 them at -80°C.

178 **2.5 Immunoblot analysis**

179 After subjecting the samples to polyacrylamide gel electrophoresis (SDS-PAGE), the proteins
180 were carefully transferred onto a nitrocellulose membrane (Bio-Rad, Cat# 1620112) of
181 dimensions (8.6 X 6.7 cm) at 100V with a constant current for 60 mins at 4°C in a Bio-Rad
182 Mini-Transfer Cell (Bio-Rad, Cat# 1703930) according to the manufacturer's specifications. The
183 nitrocellulose membrane was blocked with 3% (w/v) BSA/PBS-Tween 20 solution overnight at
184 4°C. After blocking for 14-16 hours, the membrane was washed six times for 5 mins with gentle
185 agitation at 25°C with PBS/Tween 20 buffer. The membrane was then incubated for 1 h with
186 anti-His primary mouse antibodies (1:1000) at room temperature. The membrane was then
187 washed thrice for 5 mins in PBS-Tween 20 before incubation with an HRP-conjugated anti-

188 mouse secondary antibody (1:1000) and streptactin antibody (specific to the standard protein
189 marker – Bio-Rad, Cat# 1610376; 1:1000 dilution) for 1 h at room temperature. After washing
190 the membrane six more times, the membrane was incubated with clarity western ECL substrate
191 (Bio-Rad, Cat#1705060) and imaged using a chemiluminescence imager (Syngene Pxi 4 EZ).

192 ***2.6 Reconstitution of cellulose synthase into liposomes***

193 4 mg/ml of yeast total lipid extract (Avanti polar lipids, Cat# 190000C) was taken using glass
194 Pasteur pipets in clean glass vials (Avanti polar lipids, Cat# 600460), and the chloroform was
195 entirely removed by blowing it with a stream of nitrogen gas. The vials were kept under vacuum
196 overnight in a desiccator to remove any residual organic solvent. The lipid was later solubilized
197 in 400 μ l EQ buffer containing 120 mM LDAO by vortexing vigorously and placing it in
198 running warm water until the solution got clear. 600 μ l of concentrated protein was added to this
199 mixture and incubated on ice for an hour to form mixed micelles. Meanwhile, 5g of SM2 bio-
200 beads (Bio-Rad, Cat# 1523920) were washed for 5 mins, twice with 50 ml methanol and thrice
201 with 50 ml water using a magnetic stirrer before storing them at 4°C in DI water. These washed
202 bio-beads were dried at room temperature on tissue paper/Kim wipes for 10 mins before using.
203 Dried bio-beads were added sequentially to the reconstitution mixture to prevent aggregate
204 formation. 0.35 g of bio-beads was added to the reconstitution mixture and incubated at 4°C for
205 1 h with gentle agitation. After an hour, the sample was transferred to a fresh vial containing 0.35
206 g of bio-beads, and the mixture was incubated overnight at 4°C with gentle agitation. On the next
207 day, bio-beads were allowed to settle under gravity, and the supernatant was pipetted out
208 carefully without disturbing the beads. The supernatant was then subjected to ultracentrifugation
209 at 60,000 rpm (~200,000xg) in a Beckman Coulter fixed angle rotor (TLA 100.3) for 45 mins at
210 4°C. The supernatant was discarded, and the pellet containing liposomes was washed with 1 ml

211 EQ buffer (without detergent). The suspended liposomes were subjected to ultracentrifugation
212 (~200,000xg for 45 mins at 4°C) to dilute any residual detergent. The supernatant was then
213 discarded, and the liposome pellet was resuspended in 1 ml EQ buffer (without detergent). This
214 sample was subjected to extrusion using Avanti mini extruder (Avanti polar lipids, Cat#
215 6100001EA) fitted with a 100 nm pore size filter to form uniformly sized vesicles. The extrusion
216 was performed 15-21 times before collecting the samples. The extruded sample was subjected to
217 a final ultracentrifugation step at ~200,000xg for 30 mins at 4°C to remove aggregates. The
218 supernatant was then collected, and the samples were stored at 4°C before carrying out the
219 activity assay. For long-term storage, samples were aliquoted and flash-frozen before storing
220 them at -80°C.

221 ***2.7 Cellulose synthase activity assays***

222 Standard cellulose synthase assays were set up according to Omadjela et al [26]. Twenty
223 microliters of PttCesA8- or PpCesA5 containing proteoliposomes were incubated in the presence
224 of 10 mM MnCl₂, 3 mM UDP-Glucose, in a buffer containing 20 mM Tris (pH 7.5), 100 mM
225 NaCl, and 10% (vol/vol) glycerol. After incubation at 37°C for 3 h, the samples were centrifuged
226 at 15,000 rpm for 20 mins. 10 µl supernatant was incubated with 10 µl of freshly prepared
227 nucleotide detection reagent for UDP-Glo assays (Promega, Cat#: V6961) according to
228 manufacturer's specifications. The samples were incubated at room temperature for an hour, and
229 luminescence was recorded using luminescence protocol in a Spectramax M5 plate reader. All
230 the studies were performed in triplicates, and the error bars reported are standard deviation from
231 the mean.

232

233 **2.8 Time course and kinetic studies of reconstituted cellulose synthases**

234 To analyze the time taken for the CesAs to reach saturation, the reconstituted proteoliposomes
235 were incubated with 3 mM UDP-Glucose, and 20 mM MnCl₂ for 4 h at 37°C and samples were
236 collected at regular intervals before running the UDP-Glo assay as mentioned previously.
237 Alternatively, for characterizing the kinetics of CesAs, the samples were incubated in the
238 presence of 20 mM MnCl₂ and 0–3.5 mM UDP-Glucose. A stock concentration of 300 mM
239 UDP-Glucose was used to dilute the substrate concentration in each reaction vial. After synthesis
240 for 30 mins, the reaction mixture was subjected to UDP-Glo assay as described above. All the
241 studies were performed in triplicates, and the error bars reported are standard deviation from the
242 mean.

243 **2.9 Kinetic analysis and calculations**

244 Preliminary data analysis was performed using Microsoft Excel™ to obtain UDP produced
245 (μmol/min). The data was fit to the monophasic Michaelis-Menten kinetic tool in Origin to
246 obtain V_{max} and K_m. The turnover number (k_{cat}) was calculated from V_{max} using the following
247 equation, as outlined in detail elsewhere [27]:

$$248 \quad k_{\text{cat}} = V_{\text{max}}/[E_T]$$

249 [E_T] = total enzyme concentration (in μM)

250 V_{max} = Velocity of the enzyme (μM sec⁻¹)

251 k_{cat} = turnover number (sec⁻¹)

252 Similarly, the data obtained from the time-course study was fitted using the non-linear curve
253 fitting tool in Origin. Curve fitting was done using the Levenberg-Marquardt algorithm with a
254 tolerance of 1e-9.

255

256 **3. Results and Discussion**

257 ***3.1 Heterologous expression and cell lysis for the extraction of CesAs***

258 Cellulose synthase was predicted to have seven transmembrane helices, an N-terminal Zn-
259 binding domain, a large cytosolic domain with a TED motif, and plant-conserved and class-
260 specific regions [22,23]. When the structure of the homotrimeric CesA8 from Poplar was
261 resolved using Cryo-EM, these predictions became more transparent [17]. Here, we used the
262 same set of genes reported previously, but the codon-optimized versions of PpCesA5 conjugated
263 with C-terminal 12x His-tag and PttCesA8 conjugated with N-terminal FLAG tag, and C-
264 terminal 12x HIS-tag for heterologous expression in *Pichia pastoris*. CesA genes were integrated
265 into the genome of *Pichia* under the control of the AOX1 promoter. Hence, the induction of the
266 protein was performed using methanol as an inducer. The integration of the gene and its whole
267 sequence was confirmed using the primers listed in Supplementary Table S1.

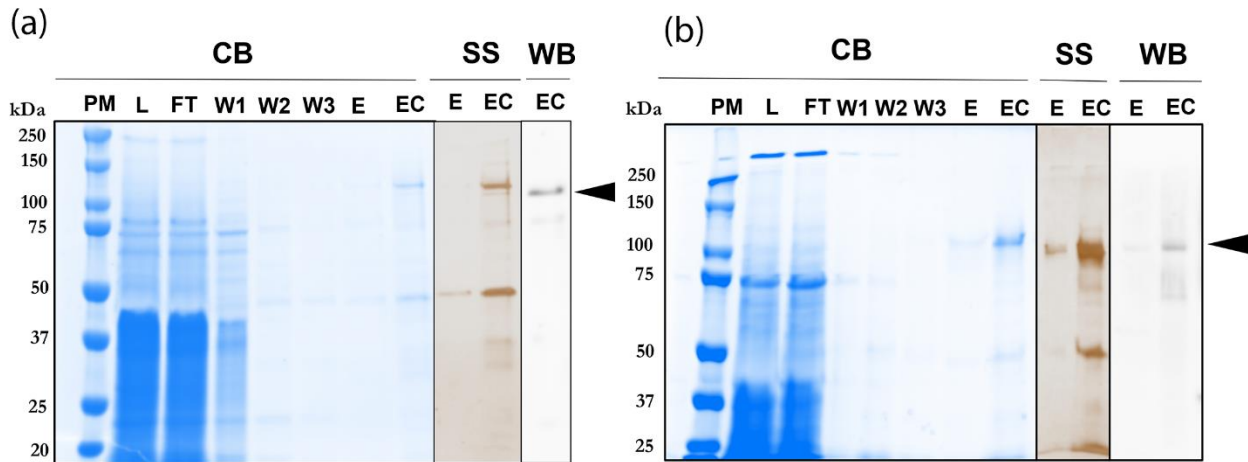
268 Membrane proteins (MP) are highly amphipathic, temperature, and shear-sensitive. Conventional
269 methods of membrane protein extraction, such as bead-beating, sonication, product entrapment,
270 and homogenization, exert a lot of physical pressure on the cells that could be detrimental to the
271 membrane protein integrity [28–30]. Moreover, methods like homogenization might also extract
272 the MPs that are folded incorrectly and not processed completely since it involves the usage of
273 whole-cell lysate [25]. During this study, the high-pressure homogenizer or the bead beating
274 method was fruitless since the former resulted in a very low yield of purified CesA proteins (Fig.
275 S2), and the latter resulted in no yield. The yield of CesAs from homogenized protein samples
276 was found to be between 25-40 µg/ml from a 1L batch. Hence, we used a slightly modified
277 *Pichia* protoplast-based extraction method that is gentler on the cells and involves chemical and

278 enzymatic treatment followed by a short solubilization step directly on protoplasts to favor the
279 extraction of the undamaged, correctly folded MPs that have been targeted to the plasma
280 membrane (Fig. 1). This could potentially help overcome the problem of decreased yields in
281 CesAs since the probability of getting correctly folded MPs is more. We observed a 3-4-fold
282 increase in the amounts of both CesA5 and CesA8 when we used the modified protoplast
283 extraction approach compared to homogenization. Only a tiny fraction of the proteins recovered
284 from the total lysate were CesAs (Supplementary Table S2).

285 ***3.2 Purification and detection of PttCesA8 and PpCesA5***

286 CesAs were purified to homogeneity in the detergent Lysofoscholine Ether 14 (LFCE14) via
287 immobilized metal affinity chromatography (IMAC). The isolated membrane fraction obtained
288 from the modified protoplast extraction method was directly used for purification. The different
289 fractions involved in the purification of PpCesA5 and PttCesA8 were observed under protein
290 detection techniques such as Coomassie and silver staining. The enriched proteins were found to
291 be immunoreactive when treated with anti-HIS antibodies (Fig. 2; Fig. S3). The final elute had
292 highly enriched PttCesA8 and PpCesA5 proteins of approximately 110 and 125 kDa,
293 respectively, when compared to a standard protein marker. A ~50 kDa band was observed under
294 SDS-PAGE analysis in both cases. Interestingly, this band did not appear when the fraction was
295 raised against the anti-HIS antibody, as mentioned in some previous reports [22,23]. Also,
296 reducing the zymolyase treatment time from 30 mins to 20 mins nearly removed the ~50 kDa
297 band observed in the case of both CesAs (Fig. S4). Longer exposure to zymolyase treatment
298 could have resulted in delicate protoplasts making it more susceptible to cell lysis and protein
299 degradation. Relative quantity and percentage purity were determined by analysis of SDS-PAGE
300 band intensities using the Image Lab software, version 6.0.1 (Bio-Rad) as mentioned elsewhere

301 [31]. The percentage purity was 75.8 and 79.2 for both CesaA5 and CesaA8. Also, the relative
302 quantity of CesAs in the purified elute compared to membrane solubilized fraction was 55.35
303 and 52.85 respectively. These values for both CesaA5 and CesaA8 are tabulated in Supplementary
304 Table S3.



305
306 **Figure 2.** Coomassie blue (CB)- and Silver (SS)-stained SDS-PAGE WB –Western Blot- raised
307 against the C-terminal His-tag of (a) PpCesA5 (b) PttCesA8. PM- Protein Marker; L- Load; FT-
308 flow-through; W1–3, wash steps 1–3; E, eluted fraction; EC, 10x Concentrated eluted fraction.
309 The black arrowheads represent the position of the purified CesaA enzymes.

310 Although Coomassie, silver staining, and immunoblotting detected the presence of HIS-tagged
311 CesAs, we wanted to confirm the presence of PpCesA5 and PttCesA8 further using LC-MS-MS.
312 The bands corresponding to the molecular weight of both PpCesA5 and PttCesA8 were excised
313 and analyzed at a tandem mass spectrometry fingerprinting facility at Rutgers. Thirty-seven
314 peptides specific to PpCesA5 and fifty-four peptides specific to PttCesA8 were identified,
315 confirming the presence of both these proteins (Supplementary Tables S4 and S5). The other
316 proteins observed using mass spectrometry were mostly contaminating proteins arising from the
317 expressing organism. None of those contaminating proteins shows any documented evidence or

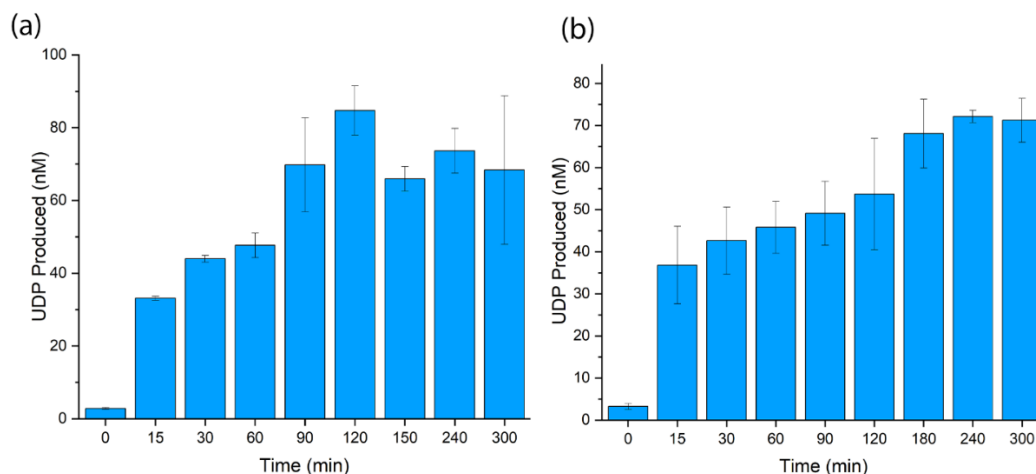
318 function in polysaccharide biosynthesis. Interestingly, no peptides matching *Pichia* β -1,3 glucan
319 synthase were observed using this method, contrary to the homogenizer based methods reported
320 previously [22,23].

321 ***3.3 Time course study shows a faster saturation for CesA5 compared to CesA8***

322 Purified CesAs were reconstituted into yeast total lipid extract liposomes using the detergent-
323 mediated liposome reconstitution method [22]. The reconstituted enzyme's catalytic activity was
324 measured in the presence of 3 mM UDP-Glucose and 20 mM Mn^{2+} . Reactions catalyzed by
325 CesAs result in the formation of UDP nucleotide that could be measured to quantify CesA
326 activity. Control reactions in the absence of proteoliposomes did not contain any UDP and the
327 background was subtracted from the obtained values. As shown in Fig. 3a and b, PpCesA5, and
328 PttCesA8 continued to produce UDP at an optimal pH of 7.5 and temperature of 37°C. The
329 catalytic activity stalls after 180 min of incubation for PttCesA8 and after 120 min of incubation
330 for PpCesA5, respectively. This could be due to the depletion in protein activity or inhibition of
331 catalytic activity by UDP produced. The maximum amount of UDP produced was 84 nM in the
332 case of PpCesA5 and 72 nM in the case of PttCesA8. A similar trend in saturation was observed
333 for the previously reported time-course synthesis studies of CesAs [22–24]. The time course
334 study for both the enzymes were observed to fit into a non-linear model as shown in
335 supplementary Fig. S5. The fitted curve shows the maximum UDP produced (P1) and the time
336 taken to produce half of the maximum UDP (P2). The curve is observed to follow a non-linear
337 trend before flattening out completely indicating saturation of product accumulation. Value of P2
338 in CesA5 is roughly double the time as that of CesA8 suggesting a faster accumulation of UDP
339 in CesA5 compared to CesA8.

340 The time-course activity data was eventually used to calculate the specific activity of the
341 proteoliposomes (Supplementary table S2). The specific activity of membrane solubilized
342 fraction was observed to be the highest (161.62 and 157.97 nmol/min/mg for CesA5 and CesA8)
343 since the UDP produced could be from other contributing fungal enzymes like β -1,3 glucan
344 synthase that utilize UDP-Glucose as substrate [32–34]. Interestingly, these enzymes were not
345 observed in neither of our purification preparations when observed under mass spectrometry
346 (Supplementary Tables S4 and S5). Hence, the UDP formed from Co-TALON elute fraction and
347 reconstituted liposomes is mostly from CesAs. The higher value observed from the reconstituted
348 liposomes could be attributed to the greater stability the lipid vesicles provide to the membrane
349 proteins than detergent micelles.

350 Compared to the bacterial cellulose synthases, the plant cellulose synthase shows almost a two-
351 magnitude difference in the specific activity according to one report [35] and similar specific
352 activity in another [36]. However, the values reported previously were obtained directly from the
353 purified and total membrane fractions and not from the functionally reconstituted liposomes. It
354 would therefore be premature on our part to make a direct comparison between the specific
355 activities across two different types of samples from two different species.

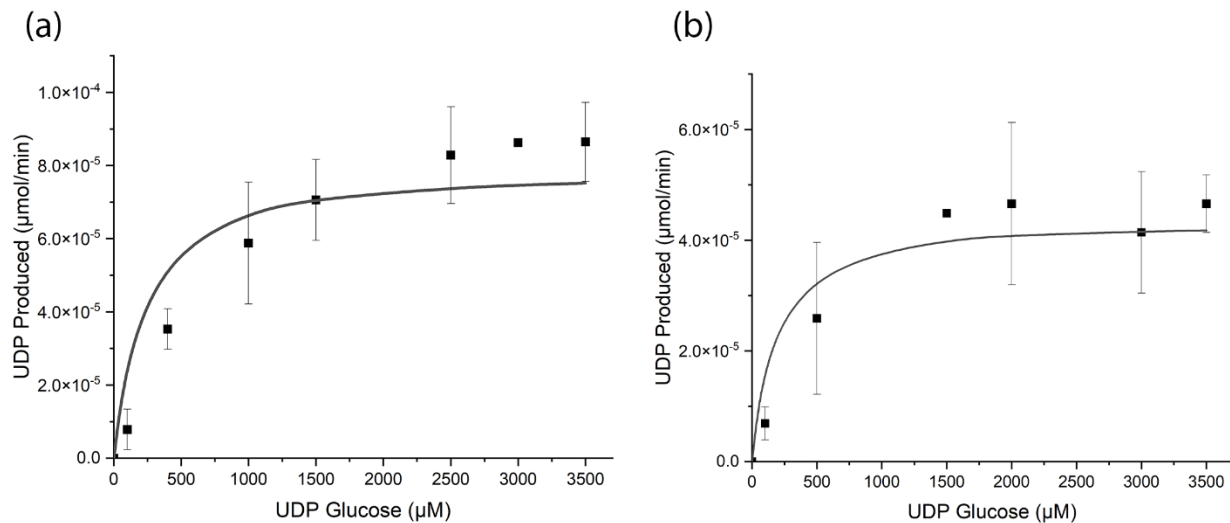


356

357 **Figure 3.** Time course of UDP biosynthesis using UDP-glucose as a substrate by reconstituted
358 (a) PpCesA5 (b) PttCesA8.

359 **3.4 Reconstituted CesA8 shows a higher substrate affinity compared to CesA5**

360 To measure the affinity of the substrate towards the enzymes, we measured the apparent K_m
361 values for both the reconstituted proteoliposomes. K_m values of PpCesA5 and PttCesA8 were
362 estimated to be 167 μM , and 108 μM , consistent with the values reported for reconstituted
363 CesAs previously [22–24]. Also, a comparable K_m value of 500 μM is observed in the case of
364 reconstituted *R. sphaeroides* BcsA [26] and 270 μM in the case of AcsA-B from *G. hansenii*
365 [37]. All the data were fit to monophasic Michaelis–Menten kinetics, and a lower K_m value in the
366 case of CesA8 suggests a greater affinity of the substrate towards this enzyme compared to
367 CesA5.



368
369 **Figure 4.** Kinetic analyses of (a) PpCesA5 and (b) PttCesA8 by titrating increasing amounts of
370 UDP-Glucose and quantification of UDP. The obtained data were fit to monophasic Michaelis-
371 Menten kinetics using Origin software, yielding a K_m of 167 μM and 108 μM .

372 The turnover number (k_{cat}) of CesA5 and CesA8 was calculated to be $1.45 \pm 0.13 \text{ sec}^{-1}$ and 0.79
373 $\pm 0.06 \text{ sec}^{-1}$ respectively. Correspondingly, the catalytic efficiency was estimated to be $0.009 \pm$
374 $0.01 \mu\text{M}^{-1} \text{ sec}^{-1}$ and $0.007 \pm 0.01 \mu\text{M}^{-1} \text{ sec}^{-1}$ for each of the enzymes. All the kinetic parameters
375 are summarized in Table 1. The reproducibility of the values was tested with two separate
376 purified enzyme preparations.

377 The k_{cat} for cellulose synthase has been published only for bacterial cellulose synthases until
378 now. Cellulose synthase from *R. sphaeroides* has been reported to have a k_{cat} of 90 sec^{-1} . Also,
379 the *Gluconacetobacter hansenii* enzyme has been reported to have a k_{cat} of $1.60 \pm 0.50 \text{ sec}^{-1}$
380 which was almost two orders of magnitude lower than that of *R. sphaeroides* [26]. Interestingly,
381 plant cellulose synthases also had k_{cat} in a similar range as that of *G. hansenii* [35,37,38],
382 meaning they also have lower values than that of *R. sphaeroides*. It is difficult to assess whether
383 the plant CesAs are slower in adding the substrate than *R. sphaeroides* since a recent study
384 involving cellulose biosynthesis at a single-molecule level shows an addition of glucose every
385 2.5 secs at room temperature [39]. Such state-of-the-art methods may be required to determine
386 the exact catalytic efficiency and turnover rate of the plant CesAs.

Enzyme	K_m (μM)	V_{max} ($\mu\text{mol}/\text{min}$)	k_{cat} (sec^{-1})	k_{cat}/K_m ($\mu\text{M}^{-1} \text{ sec}^{-1}$)
CesA5	166.95 ± 13.47	7.88E-05	1.45 ± 0.13	0.009 ± 0.01
CesA8	108.57 ± 9.78	4.31E-05	0.79 ± 0.06	0.007 ± 0.01

387
388 **Table 1.** Summary of kinetic parameters for both CesA5 and CesA8 enzymes. Experiments were
389 run in duplicates, and errors are standard deviations from the mean.

390

391

392 **4. Conclusion**

393 Primary cell walls are synthesized during cell expansion and are highly extensible and
394 incorporative. On the other hand, secondary cell walls are not extensible and typically provide
395 tensile strength and rigidity after the cell ceases expansion [40]. Primary CesAs are known to
396 physically interact both *in vitro* and *in planta*, with all secondary CesAs suggesting specialized
397 functions for CesAs in certain developmental or environmental conditions [41,42]. These CesAs
398 typically interact and form cellulose synthase complex (CSC) in higher plants [43–45].
399 Therefore, a systematic study at an enzymatic level is imperative to compare and contrast the
400 different CesAs involved in primary and secondary cell wall synthesis, respectively. In this
401 study, we have developed a simple and efficient method of CesA extraction from recombinant
402 *Pichia* protoplasts.

403 In conclusion, our work confirms that different CesAs involved in the primary and secondary
404 cell wall formation extracted using the *Pichia* protoplast-based method are catalytically active
405 and show similar biochemical and kinetic characteristics to some of the previous studies. This
406 method also results in a higher purified enzymatic yield than the homogenization-based method.
407 We also observed that some of the kinetic characteristics of plant CesAs are similar to those of
408 bacterial CesAs. The developed method also allows access to purified, membrane-bound,
409 functional CesAs that may yield structures of CesAs in the future. Such studies may eventually
410 unravel the coordination between various CesAs inside CSC in vascular and non-vascular plants.

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413

414 **Author Contributions**

415 The original manuscript draft was written by DJ and edited by SPSC. DJ and SB conducted all
416 the experiments. DJ and SPSC designed the study. All authors have given approval to the final
417 version of the manuscript.

418 **Declaration of competing interest**

419 The authors declare that they have no conflict of interest.

420 **Acknowledgement and Funding Sources**

421 This work was supported by the U.S. Department of Energy (Award Number: DE-SC0019313),
422 Rutgers Energy Institute (REI), and Rutgers University. We thank Prof. James Evans and Dr.
423 Amar Parvate for discussions and valuable inputs. We also thank Prof. Jean Baum and Prof.
424 Andy Nieuwkoop for help with access to the homogenizer and ultracentrifuge used in this study.
425 We are also grateful to Prof. Jay Sy for access to the chemiluminescence imager.

426 **Abbreviations**

427 CSC, Cellulose Synthase Complex; CesA, Cellulose Synthase; Ptt, *Populous tremuloides x*
428 *tremuloides*; Pp, *Physcomitrella patens*; UDP, Uridine Di-Phosphate; MP, Membrane Protein.

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