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High Pressure Intensification of Cassava Resistant Starch (RS3) Yields

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Abstract

Cassava starch, typically, has resistant starch type 3 (RS3) content of 2.4%. This paper shows that the RS3 yields can be substantially enhanced by debranching cassava starch using pullulanase followed by high pressure or cyclic high-pressure annealing. RS3 yield of 41.3% was obtained when annealing was carried out at 400 MPa/60°C for 15 min, whereas it took nearly 8 h to obtain the same yield under conventional atmospheric annealing at 60°C. The yield of RS3 could be further significantly increased by annealing under 400MPa/60°C pressure for 15 min followed by resting at atmospheric pressure for 3 h 45 min, and repeating this cycle for up to six times. Microstructural surface analysis of the product under a scanning electron microscope showed an increasingly rigid density of the crystalline structure formed, confirming higher RS3 content.

Keywords: Debranched cassava starch; High pressure annealing treatment; Type 3 resistant starch

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24 1. Introduction

25 Resistant starch (RS) is the non-digestible starch, which can resist the digestion by α -amylase
26 and act like dietary fibres - that helps to promote the growth of beneficial bacteria in the
27 intestine (Englyst, Kingman, & Cummings, 1992). Depending on its botanical origin and
28 process employed to form it, RS can be divided into four categories: RS1 is physically
29 inaccessible for reasons such as starch entrapment in a protein matrix or a plant cell wall (e.g. in
30 seeds and unprocessed whole grain); RS2 is raw granular starch which cannot be absorbed by
31 small intestine (e.g. those from potato and green banana); RS3 is retrograded starch, mainly
32 retrograded amylose formed during cooking and cooling processes; and RS4 is chemically
33 modified starch which is cross-linked by chemical agents and insusceptible to digest and absorb
34 in the small intestine (Chung, Donner, & Liu, 2011).

35 In general, many methods involving physical, chemical and enzymatic transformations have
36 been employed to alter the properties of starch, which enhance health attributes and/or minimize
37 defects in structure. Researchers have attempted to improve the RS yields by: 1) heat-moisture
38 treatment and annealing (Brumovsky & Thompson, 2001), 2) enzyme treatment (Vatanasuchart,
39 Tungtrakul, Wongkrajang, & Naivkul, 2010; H. Zhang & Jin, 2011), 3) combined heat/enzyme
40 treatment (Mutungi, Rost, Onyango, Jaros, & Rohm, 2009) and 4) chemical treatment (Haynes
41 et al., 2000). Since consumers are increasingly interested in natural and organic foods for health
42 and environmental reasons, employing physical and/ or enzymatic treatment appears more
43 attractive.

44 Although gelatinized starch retrogrades upon cooling and typically has a negative effect on the
45 quality of starchy foods, low-temperature storage leads to the formation of retrograded starch or
46 RS3 fraction. RS3 is of particular interest as a food ingredient because of its physical and
47 nutritional functionality and processing stability (Thompson, 2000). Several factors influence

48 the quality and quantity of RS3 in addition to storage conditions: amylose (a substantially linear
49 glucose polymer) and amylopectin (a mainly branched glucose polymer) ratio, length of
50 polymer chains or degree of polymerization (DP), retrogradation or recrystallization of amylose,
51 water content, processing steps/conditions, and the presence of lipid and other components
52 influencing gelatinization and/or the retrogradation process (Eerlingen & Delcour, 1995). In
53 addition, there are a number of studies have demonstrated that the higher the level of amylose,
54 the greater the RS3 fraction formed (Brown, Mcnaught, & Moloney, 1995). As a consequence,
55 using a debranching enzyme like pullulanase to act on gelatinized starch has become one of the
56 most important methods employed to directly cleave the branches of amylopectin and produce
57 linear α -glucan chains (Pongjanta, Utaipattanaceep, Naivikul, & Piyachomkwan, 2009;
58 Vatanasuchart et al., 2010; H. Zhang & Jin, 2011).

59 Based on studies involving physically modified starches, research has indicated that the high
60 hydrostatic pressure (HHP) plays an important role in inducing gelatinization of starches while
61 still maintaining their granular integrity (Kasemwong, Ruktanonchai, Srinuanchai, Itthisoponkul,
62 & Sriroth, 2011; Oh, Pinder, Hemar, Anema, & Wong, 2008). However, subsequent to HHP
63 treatment, a rapid retrogradation has been observed (Kawai, Fukami, & Yamamoto, 2007).
64 Additionally, a combination of pressure and temperature can create more nuclei in starches
65 (Hartel, 2001) and lead to a higher yield of recrystallized starch as a resistant starch product.
66 This treatment was used for wheat starch to produce resistant starch following several steps such
67 as annealing and storage, enzyme/acid hydrolysis and annealing-pressure cycle (Bauer, Wiehle,
68 & Knorr, 2005). It has been reported that a combination of treatments enhances the yields of RS
69 in wheat starch more than by using individual processes such as HHP or thermal treatment.

70 Native cassava or tapioca starch (*Maniho esculenta Crantz*) is one of the food ingredients
71 consisting of 17% amylose and 83% amylopectin (Breuninger, Piyachomkwan, & Sriroth, 2009).
72 Given its high degree of branching, a higher formation of RS3 fraction may be expected by

73 debranching (Mutungi, Onyango, Jaros, Henle, & Rohm, 2009; Vatanasuchart et al., 2010).
74 Furthermore, there are many methods available to improve the recrystallization of debranched
75 cassava starch, such as by annealing, autoclaving-cooling cycle and/or heat-moisture treatment
76 resulting in the rise of RS3 content (Mutungi, Rost, et al., 2009).

77 Despite its promising potential, very limited information exists within published scientific
78 literature on the effects of high hydrostatic pressure (HHP) on retrograded resistant starch or the
79 RS3 content of debranched starch, particularly from cassava or tapioca. This study explores the
80 potential of forming RS3 in the structure resulting from the recrystallized debranched cassava
81 starch, following the application of combined HHP and thermal annealing. This work also aims
82 to evaluate the use of HHP within a solvent-free environment to maximise the production of
83 RS3.

84

85 **2. Materials and methods**

86 *2.1. Materials*

87 Native cassava starch was supplied by Siam Modified Starch Co., Ltd (Thailand) in the form of
88 white powder, containing less than 14% moisture and 0.2% ash content. Pullulanase solution
89 (Sigma E2412, 1,824.68 U/ml), Trehalose (Fluka 90208), Maltotetraose (DP4 - Supelco 47877),
90 Maltopentaose (DP5 – Supelco 47876), Maltohexaose (DP6 – Supelco 47873), Maltoheptaose
91 (DP7 – Supelco 47872) were all obtained from Sigma-Aldrich Co., Ltd. (United Kingdom).
92 Resistant starch assay kit (K-RSTAR) was purchased from Megazyme International Ireland Ltd.
93 (Ireland). All other chemicals was used were of analytical grade.

94

95 *2.2. Process steps to produce resistant starch from cassava starch*

96 2.2.1. *Debranching of cassava starch*

97 Native cassava starch (**NS**) was hydrolysed by pullulanase enzyme to cleave the α 1,6 glycosidic
98 bonds at the branched points of amylopectin molecules; the method employed was adapted from
99 [Mutungi, Rost, et al. \(2009\)](#). A stock solution of pullulanase (Sigma E2412) was diluted in 20
100 mM sodium acetate-hydrochloric acid buffer (pH 5.0) to 25 U/ml before use. The starch (20 g)
101 was weighed into 250 ml polycarbonate centrifuge bottle and suspended in 140 ml of 20 mM
102 sodium acetate-hydrochloric acid buffer (pH 5.0). The starch suspension (12.5%w/w) was
103 gelatinized by autoclaving at 121°C for 15 min and cooled to 50°C. The starch gel was mixed
104 with 20 ml of 25 U/ml pullulanase solution. This mixture (11%w/w starch) was incubated at
105 50°C in a shaking water bath (Grant OLS 200, Cambridge, UK) at 100 stroke/min in linear
106 motion for 24 h. The residue was recovered after inactivating the enzyme by washing thrice with
107 chilled deionized water (temperature < 4 °C) and centrifuging for 10 mins at 3000 rpm (Sorvall
108 RC-5B Plus, Kendro, Newtown, USA). The use of chilled water for inactivating this enzyme
109 has been employed earlier by [Mutungi, Rost, et al. \(2009\)](#). The pellet was freeze-dried (Martin
110 Christ Gamma 2-16, Osterode am Harz, Germany), ground in a mortar, and sieved through a
111 mesh size of 212 μ m. This sample was termed **DS**.

112 2.2.2. *Incubation of debranched-autoclaved cassava starch*

113 The effect of incubation condition on the yields of RS3 from the debranched-autoclaved cassava
114 starch (**DAS**) was determined. A 2 x 5 x 5 factorial experiment covering starch solution
115 concentration, temperature and time, was performed and carried out in triplicates. The DS (0.5
116 g) was weighed into 30 ml glass vial and mixed with 4.5 and 2 ml deionized water to form 10
117 and 20%(w/w) concentrated solutions, respectively. The mixtures were autoclaved at 121°C for
118 15 min and cooled to 50°C. These samples were allowed to stand for 15 min at ambient
119 temperature before incubating at 4°C (in a fridge), 20, 50, 60 and 90°C (in water baths) for 0.25,

120 2, 4, 8, and 24 h. The two sets of solutions (i.e. 10 and 20%) were freeze-dried (VirTis Bench
121 Top K Series, SP Industries, Warminster, PA, USA). These samples were referred to as: **DAS-**
122 **10** (i.e. the one from 10% DS) and **DAS-20** (from the 20% DS).

123 *2.2.3. Pressurizing of debranched-autoclaved cassava starch*

124 The effect of high hydrostatic pressure annealing on the development of RS3 content was
125 investigated. The debranched starch (DS, 0.5 g) was weighed into 30 ml glass vial and mixed
126 with 2 ml deionized water and autoclaved at 121°C for 15 min to make the DAS-20. The
127 resulting gel was transferred to a polyethylene pouch; vacuum packed (Mutivac A300,
128 Wolfertschwenden, Germany), and pressurized in a high-pressure vessel (37mm diameter and
129 246 mm length) (Stansted Fluid Power type Food Lab 900, Stansted, U.K), where the
130 temperature was controlled at 60°C by using a circulating thermostatic water bath (Grant B20-
131 632, Cambridge, UK). Two sets of experiments were undertaken in the high pressure rig:

132 In the first set of experiments, the pressure applied was constant and continuous for a given
133 period of time. Packed samples of DAS-20 were pressurized at 200, 400 and 600 MPa for 0.25,
134 0.5, 1, 2, 4, 8 and 24 h at 60°C to yield a 3 x 7 factorial experiments, each performed in
135 triplicates. The samples were then unpacked and transferred to 15 ml centrifuge tube before
136 being freeze-dried. These samples were named **HPT-DAS**.

137 In the second set of experiments, the application of high pressure was intermittent or cyclic. The
138 DAS-20 samples were subjected to pressure of 400 MPa at 60°C for 15 min followed by
139 atmospheric holding for 3 h and 45 min constituting one cycle; this cycle was repeated up to six
140 times covering a total treatment period of 24 h. Samples were drawn for analysis after each
141 cycle, and RS3 contents were compared with a corresponding control sample, which was simply
142 incubated at atmospheric pressure and 60°C for the same duration of time. This set of
143 experiments therefore involved a 2 x 6 factorial performed in triplicates. The cyclic treated

144 samples, named **HPC-DAS**, were then unpacked, transferred to 15 ml centrifuge tube and
145 freeze-dried, individually.

146
147 *2.3. Measurement of chain length distribution of debranched starch*

148 High-performance anion exchange chromatography, equipped with pulsed amperometric
149 detector (HPAEC-PAD) and a CarboPac PA200 Dionex DX-600 (Dionex, Sunnyvale, CA,
150 USA) was undertaken to determine polymer chain length distribution of the debranched cassava
151 starch. The sample prepared and condition employed were adapted from Dionex corporation
152 (2004) and Mutungi, Rost, et al. (2009). Trehalose (10 mg) was suspended in 10 ml of ultrapure
153 water (UPW) and used as an internal standard. Molto-oligosaccharide standards (DP4 – DP7, 1
154 mg/ml) were prepared in 150 mM aqueous sodium hydroxide solution and instantly diluted 50-
155 fold with UPW, containing 10 μ l of internal standard stock. The standards were performed to
156 identify peak by comparing retention time of sample peaks with those of standards, and to
157 predict peaks at the higher DP7 of samples according to the linear relationship between the
158 retention time and the degree of polymerization. Debranched starch sample (DAS-20, 20 mg)
159 was weighed into 2 ml vials to which 400 μ l of 2 M aqueous sodium hydroxide was added and
160 mixed in a vortex mixer. This suspension was then diluted with 1600 μ l of ultrapure water and
161 mixed in the same vortex mixer at 4°C and 450 rpm for a further period of 24 h. A 20 μ l aliquot
162 of the solution was diluted 50-fold with 980 μ l 150 mM aqueous sodium hydroxide solution/10
163 μ l internal standard stock. All samples were filtered through a 0.2 μ m filter and 25 μ l was auto-
164 injected at 0.5 ml/min flow rate into the column. The waveform and durations applied were as
165 follows: $E_1 = 0.1V (t_1 0 s)$, $E_2 = 0.1V (t_2 0.20 s)$, $E_3 = 0.1V (t_3 0.40 s)$ (integration from 0.2 to
166 0.40 s), $E_4 = -2.0V (t_4 0.41s)$, $E_5 = -2.0V (t_5 0.42 s)$, $E_6 = 0.6V (t_6 0.43 s)$, $E_7 = -0.1V (t_7 0.44 s)$
167 and $E_8 = -0.1V (t_8 0.50 s)$. A gradient of 100 mM sodium hydroxide solution (Eluent A) and 150
168 mM sodium hydroxide solution, containing 500 mM sodium acetate (Eluent B) was used for

169 elution. Increasing concentration of eluent B from 5-40% (0- 13 min), 40-85% (13- 50 min) and
170 decreasing to 5% (50-70 min) were applied in linear gradients. Integrating area under individual
171 peaks was determined by using Chromeleon[®] version 6.6 software (Dionex). This experiment
172 was performed in triplicates.

173

174 *2.4. Determination of resistant starch*

175 The amount of resistant starch (RS) in all samples was investigated in triplicates using resistant
176 starch assay kit (Megazyme, Bray, Ireland) - an enzymatic method recommended by the
177 Association of Official Analytical Chemists (AOAC) Method 2002.02 (McCleary & Monaghan,
178 2002). The main features of this procedure are: removal of non-resistant starch by hydrolysis
179 and solubilisation using pancreatic α -amylase and amyloglucosidase (AMG), washing the
180 residue with ethanol, neutralization and enzymatic hydrolysis of RS to glucose using 2M KOH
181 acetate buffer and AMG, and measurement of RS by quantification of glucose with glucose
182 oxidase/peroxidase reagent (GOPOD). The RS was calculated as $\text{mg glucose} \times 0.9$.

183

184 *2.5. Evaluation of microstructure*

185 The surface of the resistant starch samples were scanned using scanning electron microscope or
186 SEM (S360, Leica Cambridge, UK). A small amount of dried sample was attached to
187 electrically conductive double-sided adhesive carbon disc, which was pressed on a specimen
188 stub. Gold was used to coat the sample using a sputter coater (S150B, BOC Edwards, Crawley,
189 UK). The SEM operation conditions were: working pressure $< 1.0\text{E-}4$ Torr, accelerating
190 voltage = 20 kV and working distance = 14 mm at the magnifications of 300 \times , 1000 \times and

191 3000×. The image was recorded using IScan 2000 image software (ISS Group, Manchester,
192 UK).

193

194 2.6. Statistical analysis

195 All RS percentages obtained were subjected to analysis of variance – ANOVA using PASW
196 statistics 18.0 software (SPSS, IBM, Somer NY, USA). The results were expressed as mean
197 values with standard deviation. The differences between the group mean values were established
198 at 95% confidence interval ($P < 0.05$) using Duncan's new multiple range test (DMRT).

199

200 3. Results and Discussion

201 3.1. Chain length distribution of debranched starch

202 Native cassava starch was debranched using pullulanase and the chain length distribution of
203 debranched starch was measured by high performance anion exchange chromatography
204 equipped with pulsed amperometric detector (HPAEC-PAD). According to Hanashiro et al.
205 (1996), branch chain types of amylopectin are classified by HPAEC to the group with
206 periodicity of 12 as DP 6–12, 13–24, 25–36 and $DP \geq 37$. These ranges of DP are referred to as
207 A-chains, B₁-chains, B₂-chains and B_{n(n≥3)}-chains, respectively. In the present study, a polymer
208 chain distribution between DP 4-45 was obtained and it is illustrated in Fig. 1. Most of the
209 shorter chains were removed with cold water, showing the amount of chains of $DP \leq 5$
210 remaining, to be only 0.5 ± 0.2 %. The proportion of A-chains (DP 6-12) was 23.0 ± 0.6 %. The
211 highest yield of B₁-chains (DP 13-24) was found to be 50.6 ± 1.1 %, whereas the B₂-chains (DP
212 25-36) and B₃ to B₄-chains ($DP \geq 37$) were lower at 22.0 ± 0.3 % and 3.9 ± 0.6 %, respectively.
213 These results indicate that the cassava amylopectin mainly comprises of A and B₁-chains, which

214 conforms to the literature results, even though it varies with the cultivars (Charoenkul, Uttapap,
215 Pathipanawat, & Takeda, 2006; Mutungi, Rost, et al., 2009). The average chain length is DP
216 20.5, which is similar to the values observed in previous studies for debranched cassava starch
217 (Mutungi, Rost, et al., 2009; Tester, Karkalas, & Qi, 2004). Schmiedl et al. (2000) also reported
218 that effective formation of RS3 can be produced from linear glucose chains of DP 10-35.

219

220 *3.2. Effect of debranching and autoclaving on the formation of RS3*

221 The RS contents of native starch (NS), debranched starch (DS), and debranched-autoclaved
222 starch (DAS) are presented in Table 1. After debranching, the amount of RS increases
223 drastically from $2.4 \pm 0.2\%$ in NS to $17.4 \pm 0.5\%$ in DS. These results are also consistent with
224 other studies that demonstrated debranching by using pullulanase enzyme in: 1) maize starch,
225 which increased the RS yield from 0.60 to 25.5% within 24 h (Marija, Milica, & Ljubica, 2010);
226 and 2) corn starch, where RS yield increased from 0.67 to 19.02% within 12 h (Gao, Li, Jian, &
227 Liang, 2011). This implies that the hydrolysis of α -1 \rightarrow 6 linkages in amylopectin can produce
228 more linear structures similar to the amylose chains, and/or create free A-chains of amylopectin
229 in the form of double helix and crystallite segments. These debranched structures closely pack
230 into the crystal formation as retrograded starch (RS3) during retrogradation or the annealing
231 period (Vasanthan & Bhatt, 1998).

232 Moreover, the results given in Table 1 confirm that the RS content of DS is higher than NS. It
233 may be mentioned here that there are conflicting reports in literature about the RS contents of
234 DS and NS. Mutungi, Rost et al. (2009) found that the RS content in DS (21.43g/100g) was
235 significantly lower compared to that in NS (43.96g/100g), which was also observed by
236 Vatanasuchart et al. (2010). In contrast, Charles et al. (2005) noted that the initial RS yield in
237 five native cassava starches was only 6.8–14 %. These discrepancies may be due to the different

238 botanical origin of cassava. It may also be noted that the RS fraction in NS is type 2 due to the
239 compact structure limiting the accessibility of the digestive enzyme in granular starch form,
240 whereas in DS, it is type 3 which results in retrograded polymer chains being formed in the
241 gelatinized starch (Mutungi, Rost, et al., 2009; Ozturk, Koksel, Kahraman, & Ng, 2009).

242 After autoclaving of 10 and 20 %w/w DS, the RS3 content of debranched-autoclaved starch
243 (DAS-10 and DAS-20) is significantly higher (22.0 ± 0.5 and $28.3 \pm 1.0\%$, respectively) than
244 that of DS ($17.4 \pm 0.2\%$). During the autoclaving experiment, the temperature was gradually
245 increased to 121°C , maintained steady for 15 min, and then cooled down to 50°C before taking
246 samples. The total time for this process was approximately 2 h. Thus, the RS3 forms in two
247 steps that involve starch hydrolysis during autoclaving at a high temperature, followed by
248 recrystallization during cooling. It is important to note that the proportion of RS3 is strongly
249 enhanced when starch is debranched to increase the number of linear molecules prior to thermal
250 treatment (i.e. autoclaving). A similar response was also seen in the case of debranched-
251 autoclaved wheat starch (Berry, 1986) and banana starch (González-Soto, Agama-Acevedo,
252 Solorza-Feria, Rendón-Villalobos, & Bello-Pérez, 2004).

253

254 3.3. Effect of concentration, temperature and time on the formation of RS3

255 Fig. 2 shows the proportion of RS3 in debranched-autoclaved cassava starch of two
256 concentrations (10 and 20%w/w of DS) at various temperatures (4, 20, 50, 60 and 90°C), and
257 times (0, 0.25, 2, 4, 8 and 24 h). The results suggest that there are significant interaction effects
258 ($P < 0.05$) between concentration, temperature and treatment time. The initial RS3 content of
259 debranched-autoclaved starch at the higher concentration DAS-20 ($28.3 \pm 1.0\%$ RS) is clearly
260 higher than DAS-10 ($22.0 \pm 0.5\%$ RS). Thus, DAS-20 is more effective in recrystallization due
261 to re-association of a significant amount of short linear α -glucan occurring with deionized water

262 as the plasticizer. In contrast, DAS-10 has excessive water, leading to an obstruction of the
263 intermolecular interaction between the hydrogen bonding of short chain fragments (Y. Zhang &
264 Rempel, 2012).

265 After incubating, the RS3 contents in DAS-10 and DAS-20 are found to follow a similar trend.
266 The yield of RS gradually rises from 4 to 60°C, and then drops at 90°C. Theoretically, the
267 mechanism of recrystallization in amorphous polymers consists of nucleation, propagation, and
268 maturation – which represents crystal perfection by slow growth. The nucleation rate of linear
269 glucans largely increases as the temperature decreases to the glass transition temperature (T_g) at
270 approximately -5°C, whereas the propagation rate increases as the temperature increases to the
271 melting temperature (T_m) of about 150°C (Biliaderis, 2009; Ring et al., 1987). In other words,
272 the recrystallization correlates with the molecular mobility and crystal growth rate, and can
273 occur between T_g and T_m or in the glassy state (Marsh & Blanshard, 1988). Consequently, the
274 treatment temperature at 4 and 20°C may be above and close to the T_g , which favours nuclei
275 formation, however, the crystals tend to develop slowly. Thus, the increased RS3 yield of DAS-
276 10 and DAS-20 at low temperature is not observed even after a prolonged incubation time to 24
277 h. The RS formation at 90°C also reveals a distinctively low value within 24 h at both
278 concentrations; this indicates that the temperature may be above T_m , which means the glucan
279 polymers are completely transferred into the liquid state. Therefore, there is no positional order
280 in the short chain molecules to result in the formation of RS. On the other hand, the formation of
281 increased RS3 in the case of DAS-10 and DAS-20 at 50 and 60°C, further increased with
282 treatment time. The highest RS3 content of 29.6 ± 0.5 % in DAS-10 and 36.5 ± 0.3 % in DAS-
283 20 is accomplished at 60°C after 4 h. Clearly this temperature influences orientational mobility
284 and formation of double helices to promote crystal growth for retrograded starch (Jayakody &
285 Hoover, 2008). In summary, based on the present study, the highest RS3 content is formed by
286 the autoclaving of 20% debranched starch solution at 60°C for 4 h.

287

288 *3.4. Effect of high-pressure processing and annealing on the formation of RS3*

289 Fig. 3 shows the effect of high-pressure treatments at 60°C on the yield of RS3 in DAS-20. It is
290 clear that the RS3 levels in DAS-20 can significantly improve by combining pressurizing at 400
291 MPa and annealing for various times (Fig. 3A); although at a treatment time of 24 h, the
292 increase in RS3 is not significantly different ($P > 0.05$) between 400 MPa HPT-DAS and the
293 control-annealed sample. During the first 15 min, the RS3 formation in the combined treatment
294 at 400 MPa surprisingly accelerated with a 25.7% increase (from $28.7 \pm 0.5\%$ to $36.1 \pm 0.5\%$
295 RS), whereas the control-annealed sample only had a 9.7% increase of RS (from $28.7 \pm 0.5\%$ to
296 $31.5 \pm 1.1\%$ RS). These results clearly indicate that pressuring for short times provides a
297 significantly higher initial rate of recrystallization.

298 To obtain a higher yield of RS3, DAS-20 samples were subjected to cyclic high pressure
299 annealing treatment. As the results demonstrate, cyclic pressure annealing at prolonged
300 treatment times (Fig. 3B) can further enhance the RS3 formation. Although during the first 12 h
301 the increase in RS3 was not significantly different ($P > 0.05$) between HPC-DAS and HPT-
302 DAS at 400 MPa, after four to six cycles (16-24 h) the RS3 yield of HPC-DAS was greater than
303 that of either the 400 MPa HPT-DAS and the control-annealed samples. A possible explanation
304 is that, the seed crystal formation occurs when the volume of the system decreases by increasing
305 pressure, forcing starch molecules closer together and creating more nuclei in the glassy state.
306 Although high-pressure application accelerates the nucleation rate, these nuclei are limited to
307 propagate. However, these seeds lead to the formation of large crystals when holding at
308 atmospheric pressure (lower pressure level). This scenario is similar to the high-pressure
309 crystallization of cumin aldehyde (essential oil) (Moritoki, Nishiguchi, & Nishida, 1997) and
310 lysozyme (protein) (Moritoki, Nishiguchi, & Nishida, 1995). Therefore, it implies that the

311 propagation of crystalline DAS is restricted under high-pressure but cyclic pressure annealing
312 improves the rate of propagation.

313 On the other hand, despite an increase in RS, HPT-DAS at 200 and 600 MPa were not
314 significantly different in RS3 content between the groups, and their RS contents were lower than
315 the control sample even after pressurizing for 24 h (Fig. 3A). This behaviour is probably due to
316 limiting propagation of crystalline DAS under high-pressure conditions at 200 MPa, while the
317 low level of RS3 at 600 MPa could be due to the crystalline melting of DAS. A number of
318 published papers report partial melting of the crystalline structure during compression at very
319 high pressure: for instance, at 650 MPa in polylactides (Ahmed, Varshney, Zhang, &
320 Ramaswamy, 2009); and at 740 – 1,500 MPa in several native starches, including normal corn,
321 waxy corn, wheat and potato starches (Liu, Selomulyo, & Zhou, 2008).

322 Overall, the high hydrostatic pressure conditions for producing highest amount of RS3 are
323 suggested to be concurrently pressurizing DAS-20 at 400 MPa and annealing at 60°C for 15 min.
324 Clearly these conditions significantly reduce the process time, from 8 h (single incubating at
325 60°C) to only 15 min with the same RS content generated (36% RS) as in the case of the
326 conventional process. The highest RS yield was obtained after six cycles (24 h of total time) of
327 pressure alternating between 400 MPa and atmosphere under the temperature of 60°C ($41.9 \pm$
328 0.5% RS). Bauer et al. (2005) also noted increase in RS content from 2 to 12 % in the case of
329 wheat starch when the pressure was increased to 500 MPa for 15 min every 24 h, over a period
330 of 10 days. Thus, it is important to note that high-pressure application on debranched starch
331 results in greater enhancement of RS compared to high-pressure treated native starch that still
332 maintains its granular form.

333

334 *3.5. Microstructure of RS3*

335 The effects of: debranching and autoclaving on cassava starch (Fig. 4), concentration,
336 temperature and time (Fig. 5A), and high-pressure treatments on autoclaved samples (Fig. 5B)
337 were monitored using scanning electron microscopy (SEM). Fig. 4A shows the native starch
338 (NS) in a granular form. After debranching, the NS loses its granular structure, and then the
339 surface of DS appears more fluffy which indicates that the glucan polymers only reassociate
340 loosely (Fig. 4B). After autoclaving the DS, a densely packed surface region is evident in DAS
341 (Fig. 4C). In Fig. 5A, it is apparent that the DAS-20 incubation at 60°C for 4 h exhibits a
342 smoother area than DAS-10. When subjecting DAS-20 to high pressure annealing treatments,
343 Fig. 5B demonstrates that HPC-DAS after six cycles (24 h of process time) has a more densely
344 packed surface than HPT-DAS. It clearly shows less porosity and a smoother surface area,
345 which would yield greater resistance to enzyme digestion and increasing the RS content. The
346 overall microstructural observations show that RS3 content increases as the rigid dense
347 crystalline structure increases.

348

349 **4. Conclusion**

350 The process employed plays an important role in accelerating retrogradation and the
351 transforming of native starch into RS3. In this study, the debranching step gave more linear
352 glucans and the autoclaving step aggregated these to increase crystallinity. High pressure
353 annealing subsequently accelerated RS formation within 15 min, in contrast to atmospheric
354 annealing (single incubation) which required up to 8 h to result in the same yield of RS3. Thus,
355 process times can be drastically reduced using high pressure annealing. Yields of RS3 fraction
356 can be further increased following cyclic high pressure annealing of debranched-autoclaved
357 starch. The highest RS yield was obtained after applying six cycles (24 h of process time) of
358 pressure, each alternating between pressure application (400MPa/60°C/15 mins) to accelerate

359 the nucleation rate of starch crystallization, and incubation (atmospheric pressure/60°C/3h 45
360 mins) for crystal propagation (**41.9 % RS**). These conditions gave the highest yield of RS3 from
361 a 20% w/w solution of debranched-autoclaved starch. Thus, the high pressure annealing
362 treatment is highly promising to increase RS yield. In addition, this method intensifies the
363 formation of RS by physical modification (i.e. without using solvents), which is safer for food
364 industry use.

365

366 **Acknowledgments**

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368 the Siam Modified Starch Co., Ltd, Thailand for providing the raw material (native cassava
369 starch).

370

371 **Reference**

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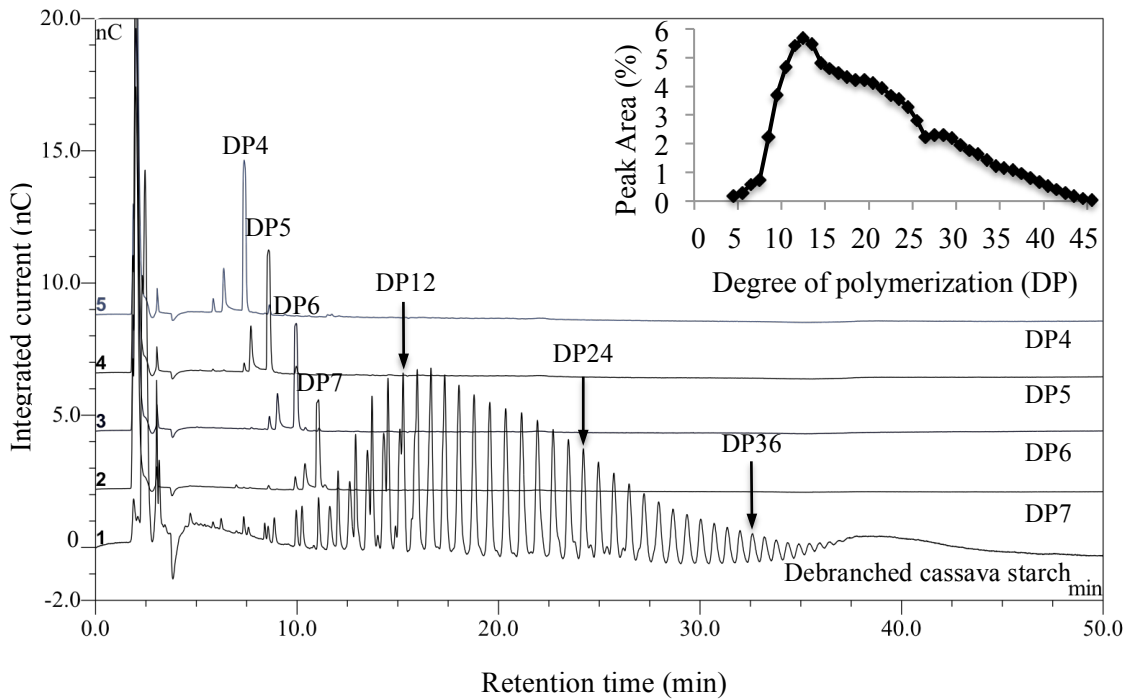


Fig. 1 Polymer chain length distribution of debranched amylopectin of cassava starch (DS) using high performance anion exchange chromatography with pulsed amperometric detector (HPAEC-PAD). The DP4-45 is shown in the chromatogram. DP4-7 peak labels indicate the DP from molto-oligosaccharide standards. The inset shows the relative peak area for the individual DP from the mean of three independent measurements.

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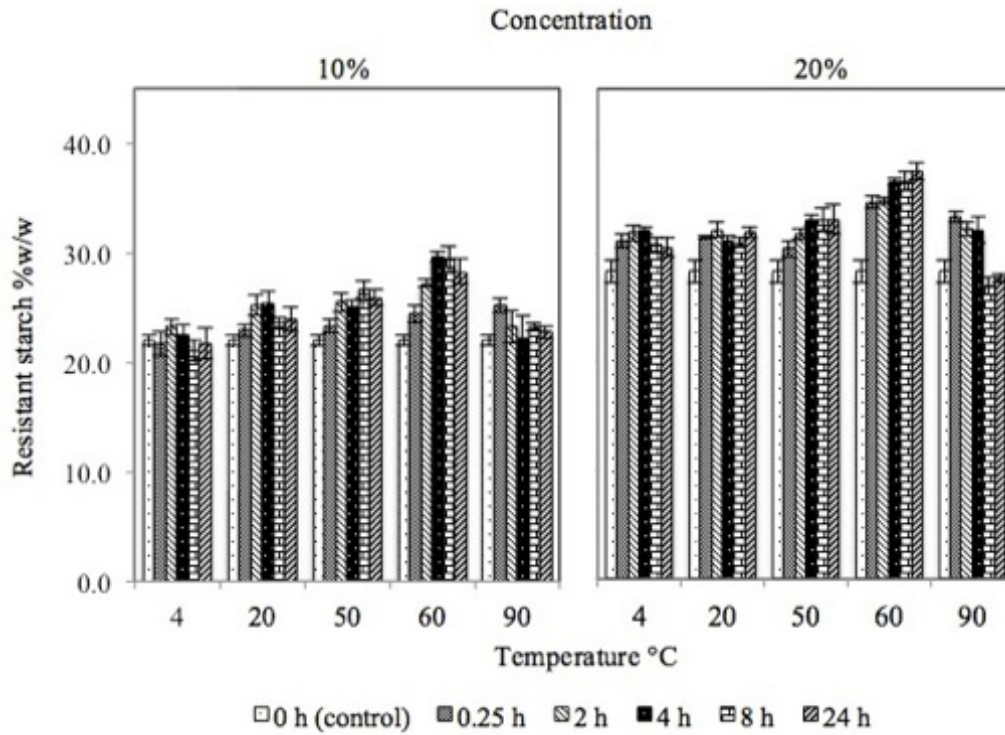


Fig. 2 Effect of concentration (10 and 20%w/w of debranched starch), temperature (4, 20, 50, 60 and 90°C) and time (0, 0.25, 2, 4, 8 and 24 h) on yield of RS3 (%w/w) of debranched-autoclaved cassava starch (DAS).

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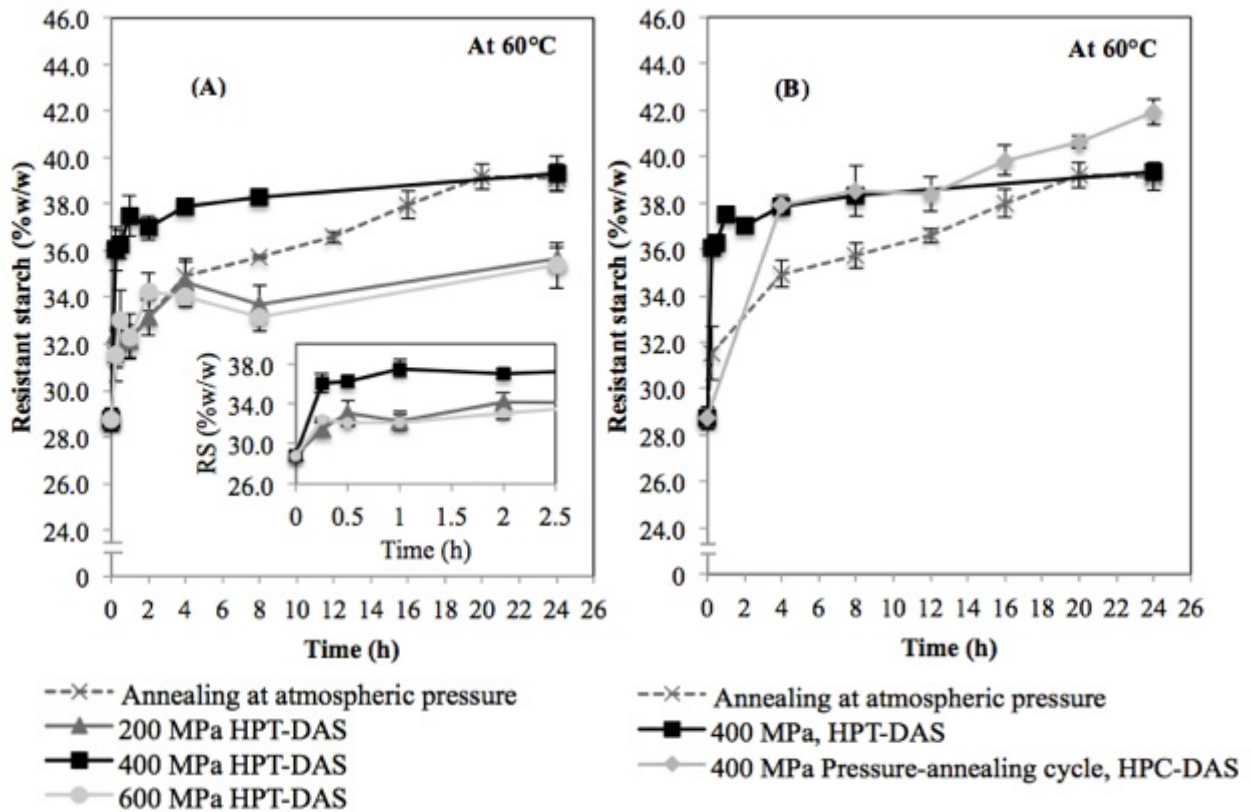
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550 **Fig. 3** Effect of high-pressure incubation on yield of RS3 from debranched-autoclaved starch

551 (DAS-20, 20% w/w of debranched starch) (A) high pressure (200, 400 and 600 MPa) at 60°C

552 for different times (0.25, 0.5, 1, 2, 4, 8 and 24 h): HPT-DAS. The inset to A shows increasing

553 RS3 content on an expanded scale. (B) high pressure incubation at 400 MPa and 60°C for 15

554 min followed by atmospheric holding at the same temperature for 3h 45 min, repeating this

555 cycle for up to six times: HPC-DAS. Figure B also shows RS content comparison among

556 control treatment (atmospheric annealing at 60°C), 400 MPa HPT-DAS and 400 MPa HPC-

557 DAS, at any treatment time.

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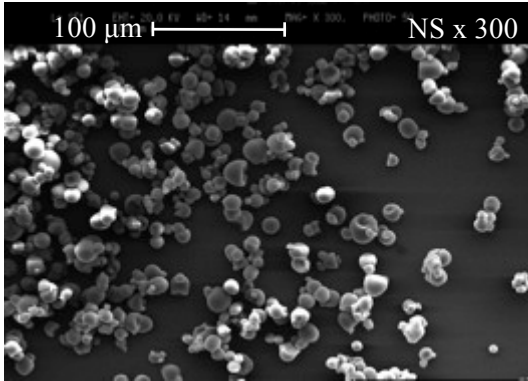
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(A)

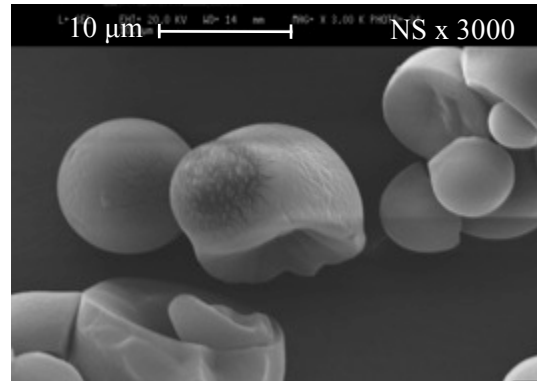
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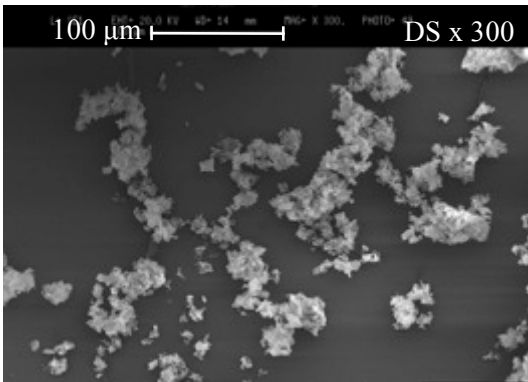
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(B)

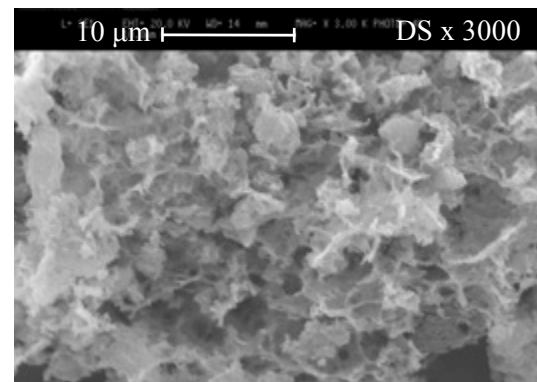
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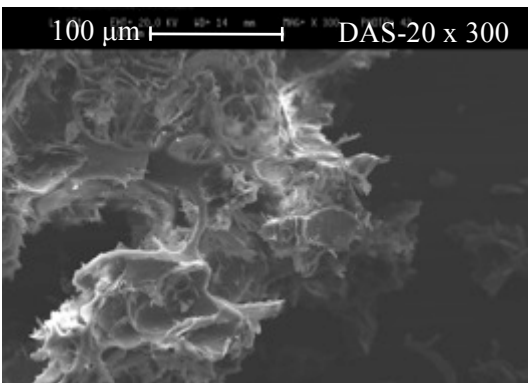
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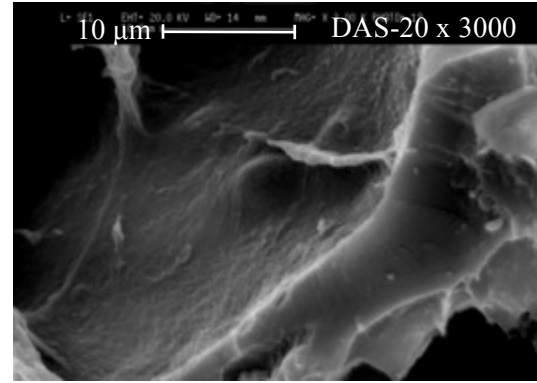
(C)

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575 **Fig. 4** Scanning electron micrographs at magnification of 300 × and 3000 × of (A) native

576 cassava starch: NS), (B) debranched starch: DS, and (C) debranched-autoclaved starch at

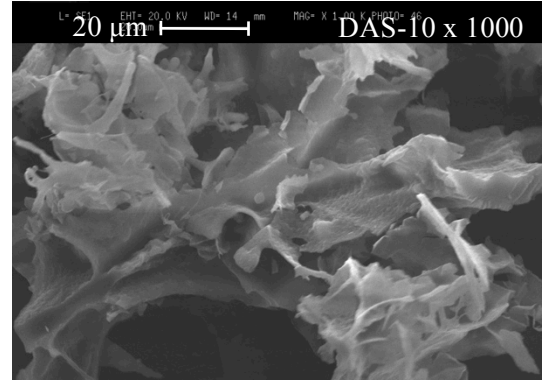
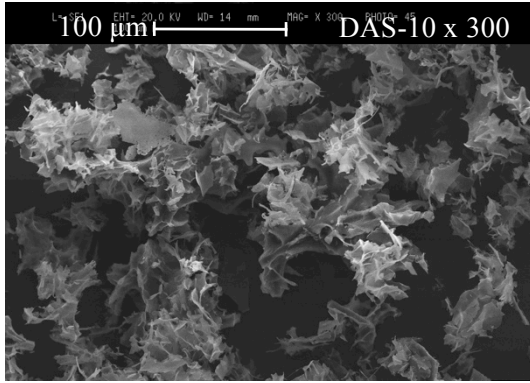
577 20%w/w of DS: DAS-20

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(A1)



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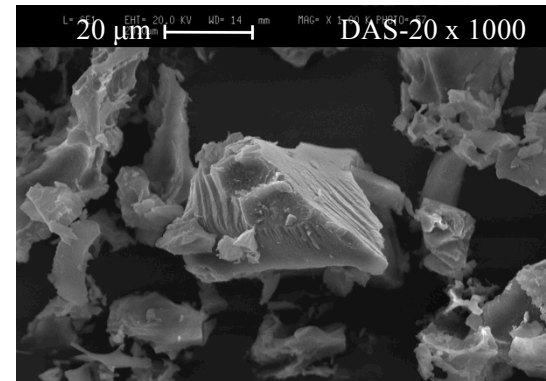
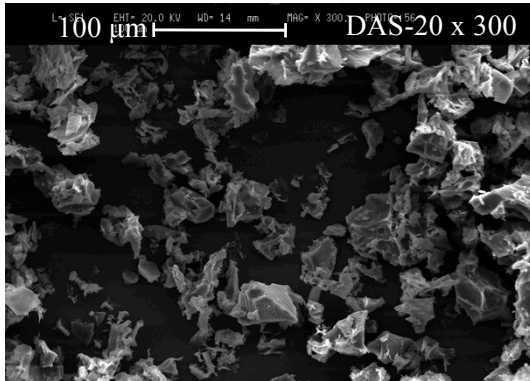
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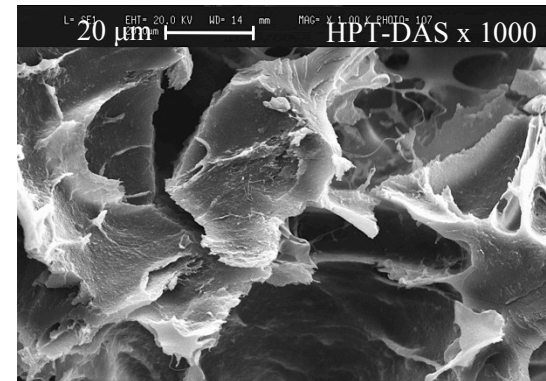
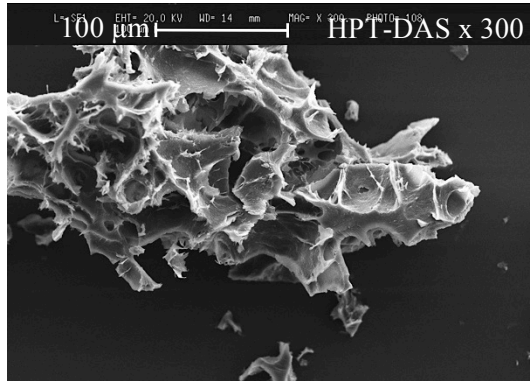
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(B1)



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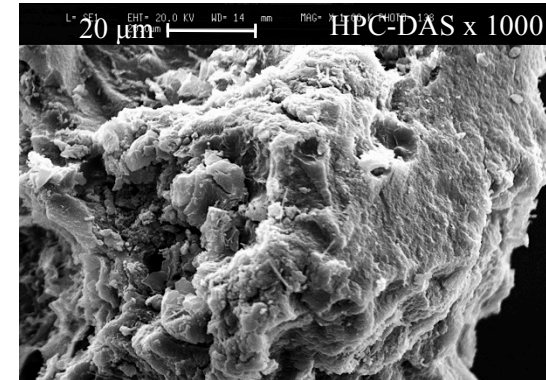
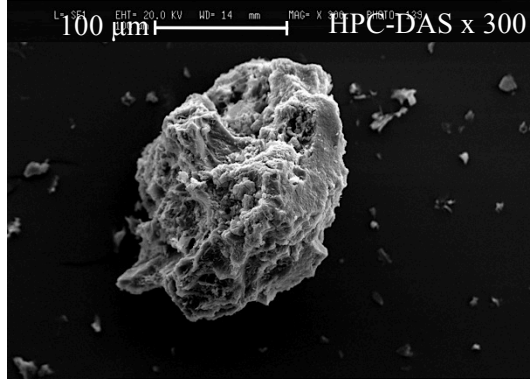
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598 **Fig. 5 (A)** Scanning electron micrographs at magnification of $300\times$ and $1000\times$ after incubation
599 at 60°C for 4 h of (A1) debranched-autoclaved starch at 10%w/w of DS: DAS-10, and (A2)
600 debranched-autoclaved starch at 20%w/w of DS: DAS-20. **(B)** Scanning electron micrographs at
601 magnification of $300\times$ and $1000\times$ of debranched-autoclaved starch at 20%w/w of debranched
602 starch after applying high pressure (B1) high pressure annealing treatment at 400 MPa and 60°C
603 at a treatment time of 24 h: HPT-DAS, and (B2) cyclic high pressure annealing at 60°C after six
604 cycles with pressure swinging between 400 MPa for 15 min and atmosphere for 3 h 45 min -
605 over 24 h: HPC-DAS.

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622 **Table 1:** Effect of debranching and autoclaving on RS3 content of cassava starch

Samples	Resistant starch (%)	Starch sources	References
NS	2.4 ± 0.2 ^a	Siam Modified Starch Co. Ltd,	
DS	17.4 ± 0.5 ^b	Thailand	
DAS-10	22.0 ± 0.5 ^c		
DAS-20	28.3 ± 1.0 ^d		
NS	43.9	Kenya Industrial Research and	Mutungi, Rost, et al., 2009
DS	21.4 ± 2.7	Development Institute, Kenya	
NS	58.2 ± 1.3	Taiwa Public Co., Ltd,	Vasanthan & Bhattya, 1998
DS	13.0 ± 1.3	Thailand	
NS	6.8 – 14.0	5 cassava genotypes (Rayong2, Rayong5, KU50, Hanatee and YOO2), Thailand	Charles, Chang, Ko, Sriroth, & Huang, 2005

623 ^{a-d} Mean ± standard deviation followed by the different superscripts are significantly different ($P < 0.05$). NS:

624 Native starch; DS: Debranched starch; DAS-10: Debranched-autoclaved starch at 10%DS; and DAS-20:

625 Debranched-autoclaved starch at 20%DS