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1 **Glutamic Acid Independent Production of Biofloculants by *Bacillus subtilis* UPMB13**

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23

24 **Abstract** *Bacillus subtilis* UPMB13 was found to be an L-glutamic acid independent producer of
25 extracellular polymeric substances (EPS) with bioflocculation properties. Optimum production of the
26 bioflocculant was found to be at the early stage of cell propagation of 24-72 h of fermentation. At a
27 limited nutrient input of 100 mL tryptic soy broth, the flocculating activities were found to be negatively
28 correlated ($p<0.01$) with growth as it continued to decline after 72 h, while cell growth proliferated
29 further. Ample nutrient supply may prolong bioflocculant production with flocculating activities of 90%
30 and higher, while excess oxygen supply may promote rapid growth that can lead to poor flocculation due
31 to the re-use of the bioflocculant as a substitute for food during starvation. Bioflocculant production
32 occurred at best at 25-30°C incubation temperature and at the initial pH medium of 7 to 8. The
33 bioflocculant was proven to be extracellularly produced as the broth and the supernatant possessed the
34 ability to flocculate the suspended kaolin particles. Bioflocculant productions by UPMB13 were
35 hereditarily stable among succeeding progenies hence, proving genetic competency. About 0.90 g of
36 purified bioflocculant were collected from 1 L culture broth of UPMB13 under the optimized
37 fermentation conditions.

38

39 **Keywords:** *Extracellular polymeric substance (EPS); biopolymer; γ -PGA; de-novo; kaolin assay*

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41

42 **1 Introduction**

43 Flocculation is a process of agglomerating suspended particles aided by compounds known as flocculants
44 commonly used in water treatment applications (Devesa-Rey et al. 2012). Among these flocculants,
45 bioflocculants have been identified as the emerging alternative to the conventional chemical flocculants
46 due to their biodegradability and environmentally benign characteristics (Muthulakshmi et al. 2013).
47 Bioflocculants are essentially extracellular polymeric substances (EPS) synthesized mostly by bacteria as
48 they are ubiquitous in nature.

49 Production of biopolymeric compounds by bacterial strains could be unpredictable even under
50 rigorously maintained culture conditions (Bajaj and Singhal 2011a). Stressful culture conditions may
51 induce and improve extracellular polymeric substances production by microorganisms (Yu et al. 2015).
52 However, definition of stressful culture conditions are species dependent and may vary for different
53 microorganisms. Salehizadeh and Yan (2014), reviewed on the production of biopolymeric flocculants by
54 microorganisms and stressed that the major criteria influencing bioflocculant performances are culture
55 conditions, including but not limited to culture time, initial pH and temperature, aeration rate, shaking
56 speed and ionic dependency. Optimization of these factors was proven to enhance the yield, quality and
57 the performances of the bioflocculants produced.

58 One of the most widely studied EPS produced by *B. subtilis* strains is the poly- γ -glutamic acid
59 (γ -PGA) (Bajaj and Singhal 2011a). γ -PGA productions typically rely on the input of L-glutamic acid
60 supplement in the culture media. Some exceptional cases reported on γ -PGA production in the absence of
61 any glutamic acid additions (Zhang et al. 2012). Notably, not all γ -PGA produced were reported as
62 potential bioflocculants.

63 This paper discusses the production of a novel bioflocculant by a locally isolated rhizobacterium;
64 *B. subtilis* UPMB13. It aims to i) optimize factors which affect the growth and bioflocculant production
65 by the strain, ii) determine the distribution of the bioflocculant produced in different culture components,
66 and iii) determine genetic competency of UPMB13 in bioflocculant production. All factors will provide
67 thorough consideration for determining the optimum culture conditions for continuous production of high
68 performing bioflocculants by *B. subtilis* UPMB13 for future studies in suspended solids treatment
69 applications.

70 **2 Materials and Methods**

71 **2.1 Biofloculant-producing bacteria**

72 *B. subtilis* UPMB13, isolated from oil-palm root, obtained from the culture collection of the Soil
73 Microbiology Laboratory, Department of Land Management, Universiti Putra Malaysia (Amir et al. 2003;
74 Zulkeflee et al. 2012) was selected based on the morphological characteristics of mucoid and ropy
75 colonies produced when grown on tryptic soy agar (TSA). These characteristics were used as basic
76 identification for potential biofloculant-producing bacteria (Wong et al. 2012). The strain was verified
77 using Basic Local Alignment Search Tool (BLAST) program analysis based on the 16S ribosomal
78 ribonucleic acid (rRNA) gene sequencing and through biochemical identification test (BBL Crystal
79 Gram-Positive ID System) which proved the strain as *B. subtilis* at 99% similarity.

80 Batch cultures of *B. subtilis* UPMB13 in tryptic soy broth (TSB) were prepared to produce the
81 biofloculants in broth form. About 50 mL seed culture of UPMB13 in TSB were prepared and incubated
82 for 24 h on an orbital shaker (150 rpm) at room temperature. Centrifuged cells (4000 rpm for 10 min)
83 from the seed culture were then washed three times with phosphate buffer solution (PBS) and once with
84 sterile TSB before inoculation into a fresh 100 mL TSB and incubated again. Viscous TSB bearing the
85 strain attained indicates bacterial growth and biofloculant production in the culture. The culture broths
86 were then used directly as the biofloculant source in the flocculation assays (Aljuboori et al. 2013).

87 **2.2 Flocculation assay**

88 Flocculation assays using kaolin clay as the suspended particle were conducted according to the methods
89 described elsewhere (Zulkeflee et al. 2012). The flocculation activities were expressed in percentage
90 clarity of the upper phase of the kaolin suspensions after treatment by the biofloculant and by visual
91 assessment; either present or absent, of the kaolin flocs formed stimulated by the presence of the
92 biofloculant.

93

94 **2.3 Production factors**

95 **2.3.1 L-glutamic acid dependency**

96 Requirement for additional L-glutamic acid supplement for bioflocculant production by UPMB13 were
97 determine by preparing batch cultures of 100 mL TSB with treatments of; L-glutamic acid (LGA) at 20
98 g/L (Bajaj and Singhal 2011b), L-glutamic acid and citric acid (CA) at 10 g/L (Chen et al. 2005), and
99 solely TSB as the control treatment. All cultures with respective treatments were incubated on an orbital
100 shaker at 130 rpm for 48 h at 25°C.

101 **2.3.2 Timeline of bioflocculant production**

102 Optical density measurements with a spectrophotometer at 660 nm were conducted to determine the
103 growth of UPMB13 for 5 days. Percentage flocculating activities of the bioflocculants produced by
104 UPMB13 were measured every 6 h until 78 h, followed by 12 hourly intervals until 120 h of incubation.

105 **2.3.3 Effect of culture medium ratio on bioflocculant production**

106 Batch cultures of UPMB13 in varying volume of TSB (100 mL, 250 mL and 500 mL) were prepared by
107 inoculating 1 mL of the seed culture into each respective volume of TSB. Percentage flocculating activity
108 measurements were done at 12 hourly intervals for 120 h.

109 **2.3.4 Effect of incubation speed on bioflocculant production**

110 Oxygen fluxes were introduced by means of varying incubation speed on an orbital shaker. Five speeds
111 were selected: 100, 150, 200, 250 and 300 rpm. Percentage flocculating activities were measured every 24
112 h for three days to observe the effect on bioflocculant production.

113 **2.3.5 Effect of initial pH and incubation temperature on bioflocculant production**

114 The initial pH of the culture media, adjusted with either HCl (1 N) or NaOH (1 N), were prepared for pH
115 5.0, 6.0, 7.0 and 8.0 (Su et al. 2012). Observations of the flocculating performances were done directly
116 after 24 h to avoid further pH changes. Two ranges of incubation temperature were selected, namely; 25-
117 30°C (Patil et al. 2009) and 37-40°C (Bajaj and Singhal 2011b) provided by an incubator shaker.
118 Percentage flocculating activities were measured at 24, 48, 72 and 96 h of incubation.

119

120 **2.4 Determination of bioflocculant source among culture components**

121 The source of bioflocculant produced among the culture components; broth, cell and supernatant, was
122 determined. The cultured cells and cell-free supernatant were separated from the cultured broth by
123 centrifugation at 4000 rpm for 10 min. The supernatant was then removed and put aside as the
124 bioflocculant source for the cell-free supernatant. The centrifuged cells were washed with phosphate
125 buffer solution (0.85%) to create a cell suspension and again centrifuged before serving as the
126 bioflocculant source for the cells.

127 **2.5 Genetic competence of *B. subtilis* UPMB13**

128 UPMB13 genetic competence for continuous bioflocculant production was investigated. Each
129 representative generation was prepared by sub-culturing TSA plate cultures of UPMB13; with the first
130 generation being the one directly sub-cultured from the parent stock agar, until the 10th generation. Batch
131 cultures of 100 mL TSB of each generation were then prepared and the percentage flocculating activities
132 were measured.

133 **2.6 Extraction and purification of the bioflocculant**

134 The culture broth of UPMB13 was first tested for flocculating activity through the kaolin assay. When the
135 flocculating activity of the culture broth achieved 90% and above, the culture broth was then centrifuged
136 at 8000 x g for 10 min at 4°C to separate the cells. The remaining supernatant was then added to 2
137 volumes of ice cold ethanol and left overnight at 4°C to precipitate the bioflocculant. The precipitated
138 bioflocculant was then collected by centrifugation at 12000 x g for 15 min at 4°C and re-suspended in
139 ultra-pure water. Further impurities were separated via dialysis against ultra-pure water with minimum
140 three times water change in 24 h at 4°C. The resulting dialyzed suspension was then lyophilize to collect
141 the pure bioflocculant.

142 **2.7 Surface morphology imaging**

143 The surface morphology of the freeze-dried purified bioflocculant was observed using a scanning electron
144 microscope (SEM) Carl Zeiss EVO-MA10 (Canada). The samples attached to carbon stubs were gold-
145 coated (Nwodo and Okoh 2012) twice and examined using the microscope at an accelerating voltage of

146 20.0 kV. Additionally, the surface morphology of the kaolin particles both before and after flocculation
147 were also scanned and observed.

148 **2.8 Data Analysis**

149 For each experiment, a minimum of three replicates of UPMB13 cultures were prepared. Descriptive
150 statistics for pattern and trend observations were determined with the mean and standard deviation values
151 measured. Significant differences were analyzed through analysis of variance (ANOVA) at 0.05
152 confidence level. Additionally, where applicable, repeated measures ANOVA were used for data
153 measured repeatedly in a timely basis.

154

155 **3 Results and Discussion**

156 **3.1 L-glutamic acid independent production of bioflocculants**

157 Both L-glutamic acid and the citric acid inhibited UPMB13 growth, as the culture broths with the
158 supplements were observed to be clear and translucent after 48 h of incubation, suggestive of the absence
159 of bacterial growth. In comparison, the control treatment without any added supplement were detected to
160 be naturally viscous after 48 h of incubation (Table 1).

161 The upper phase of the treated kaolin suspension with the supplemented broth were observed to
162 be clear, hence producing a measured flocculating activities of 71.7% and 27.7% for the LGA and the
163 LGA+CA treatment, respectively. However, no floc formation was detected in both treatments (Table 1).
164 In contrast, the control TSB media induced flocs formation with percentage flocculating activities
165 measured at 81.7% ($p<0.05$). Charge de-stabilization of the kaolin particles might occur in the treated
166 system by the LGA and LGA+CA treatments which explains the percentage flocculating activities
167 measured, despite the absence of the bioflocculant.

168 Productions of γ -PGA by *B. subtilis* strains are usually L-glutamic acid dependent, while L-
169 glutamic acid independent strains were described to be relatively unknown and thus less reported (Shih
170 and Van 2001). Some of the L-glutamic acid independent bacteria reported to produce γ -PGA through the
171 *de novo* production pathway (without supplement) includes *B. subtilis* TAM-4 (Ito et al. 1996), *B. subtilis*
172 C1 (Shih et al. 2005) and *B. subtilis* C10 (Zhang et al. 2012). Among the studies on the production of γ -

173 PGA by *B. subtilis* strains that were reported as bioflocculants (Yokoi et al. 1995; Yokoi et al. 1996; Wu
174 and Ye 2007; Wang et al. 2008; Bajaj and Singhal 2011b; Bhunia et al. 2012) none were *de novo*
175 producers of γ -PGA. Other γ -PGAs produced through *de novo* pathways (Ito et al. 1996; Shih et al. 2005;
176 Zhang et al. 2012) have not been reported as potential bioflocculants. In two rare cases, γ -PGA had been
177 reported to be produced by *Bacillus* strains either with or without (*de novo*) the presence of glutamic acid
178 (Mahmoud 2006; Xu et al. 2005). Therefore, *B. subtilis* UPMB13 is considered a novel *de novo* producer
179 of bioflocculants as compared to other cases reported in the literature.

180 Production of EPS by *B. subtilis* strains has been reported to co-dependently rely on citric acid
181 apart from the L-glutamic acid supplement (Bajaj and Singhal 2011a). Naturally, *B. subtilis* strains can
182 utilize citric acid as an organic acid carbon source for growth. However, UPMB13 were notably different
183 as citric acid supplement inhibited its growth. Xu et al. (2005) reported similarly for the strain *B. subtilis*
184 NX-2 which could not incorporate citric acid during growth for γ -PGA production.

185 Hence, *B. subtilis* UPMB13 was concluded to be a *de novo* producer of bioflocculants in the
186 absence of L-glutamic acid or citric acid supplements during growth.

187 **3.2 Bioflocculant production during growth**

188 Time course for bioflocculant productions by *B. subtilis* UPMB13 are as depicted in Figure 1.
189 Bioflocculant presence at the early stage of UPMB13 growth was reflected by the 87.2% flocculating
190 activities measured and visible flocs formation observed at 24 h of incubation. The flocculating activities
191 maintained above 80% between 24-72 h ($p>0.05$). This suggests that bioflocculant production by
192 UPMB13 occurred parallel to its logarithmic growth rate. However, between 78 h to 120 h, decrements in
193 flocculating activities were observed, although UPMB13 growth continued to proliferate. The relationship
194 between growth of UPMB13 and bioflocculant production was analyzed using Pearson Product Moment
195 correlation. There is a strong negative relationship ($R = - 0.787$) between flocculating activities of the
196 bioflocculant with growth ($p<0.01$).

197 Okaiyeto et al. (2015) reported that the decrease in flocculating activities after 72 h of incubation
198 could be due to deflocculating enzymes excreted by the strain during death phase. However, in this study,
199 the growth of UPMB13 was observed to be maintained up to 120 h while flocculating activities were
200 declining. Therefore, it was hypothesized that the limited nutrient supplied (100 mL) had led to the uptake

201 of the bioflocculant already present in the culture by the cells as alternative food for growth, while
202 productions of new bioflocculants were inhibited by the stressful conditions created by the scarcity of the
203 food. This theory was proven by the results in the next section.

204 **3.3 Effect of culture media ratio on bioflocculant production**

205 Consumption of the bioflocculants excreted into the culture media as substitute food source for growth
206 was hypothesized to be the reason for the low flocculating performances observed in the later growth
207 stage in Figure 1. This theory was further tested by varying the ratio of culture media used to the
208 inoculum and the results are as portrayed in Figure 2.

209 Both the 250 mL and 500 mL ($p>0.05$) cultures have significantly higher flocculating
210 performances as compared to the 100 mL culture of UPMB13 ($p<0.05$) after prolonged fermentations.
211 With ample nutrients provided by the 250 mL and 500 mL culture, bioflocculant productions by
212 UPMB13 had sustained until the 120 h as the percentage flocculating activities measured remained
213 around 90%. Contradictorily, the 100 mL culture experienced the normal decrease in flocculating
214 performances with prolonged growth. Hence, it was proven that the bioflocculants already present in
215 culture media excreted earlier were being taken up by the strain to support further growth while no new
216 bioflocculants were being produced in the stressful conditions of limited nutrients. It was reported by
217 Kimura et al. (2004) that excreted EPS may be a source of food during starvation in the late stationary
218 microbial growth phase.

219 **3.4 Effect of oxygen fluxes on bioflocculant production**

220 Biopolymers yield in aerobic microbial processes depends greatly on oxygen fluxes provided through
221 agitation and aeration during fermentations (Richard and Margaritis 2003). Figure 3 illustrates the
222 flocculating performances of UPMB13 cultures subjected to different shaking speeds provided during
223 incubation. Flocculating activities $>80%$ ($p>0.05$) were recorded at the optimal incubation speeds of 100
224 to 200 rpm. Significantly poorer flocculation were observed for cultures agitated at speed higher than 200
225 rpm ($p<0.05$).

226 Rapid bacterial growth at higher speed may result in the scarcity of food resources and led to the
227 re-uptake scenario of the excreted bioflocculant as alternative source for food. Excessive oxygen due to

228 rapid agitations may also inhibit biopolymeric excretions as concentration of dissolved oxygen affects
229 microbial nutrient absorption and enzymatic reaction for EPS productions (Su et al. 2012). Furthermore,
230 inhibition of production could also be due to the rapid conversion of carbon source to carbon dioxide
231 resulting from the increase in growth and respiration which led to a decrease in biopolymeric productions
232 (Patil et al. 2010.).

233 **3.5 Effect of initial pH on bioflocculant production**

234 Optimum initial pH for bioflocculant productions by UPMB13 were at pH 7.0 to 8.0 with percentage
235 flocculating activities measured >75% ($p>0.05$) (Figure 4). Although the growth of UPMB13 were
236 observed to be higher at pH 5.0 and 6, however, flocculating performances measured were significantly
237 lower ($p<0.05$). The unfavorable acidic nature of the culture media at these pH ranges might create
238 stressful culture conditions that lower bioflocculant excretions by the strain (Ntsaluba et al. 2013).

239 As bioflocculant production occurs parallel with growth, naturally the optimum pH for
240 production would be similar to the pH that induces bacterial growth. However, for UPMB13, the initial
241 pH of the culture media had more influence on bioflocculant production despite the level of growth.
242 According to Prasertsan et al. (2008), the synthesis of the enzymes that are responsible for EPS excretion
243 are pH dependent. Furthermore, it was reported that nutrient assimilation and enzymatic response of
244 microorganisms for bioflocculant production may be affected by initial pH of the culture media
245 (Aljuboori et al. 2013).

246 **3.6 Effect of incubation temperature on bioflocculant production**

247 Bioflocculant production were proven feasible in both temperature range with significantly different
248 flocculating performances observed ($p<0.05$). The 25-30°C temperature range was proven optimum for
249 both growth and bioflocculant production. In comparison, at higher temperature range of 37-40°C, the
250 growth and the flocculating performances of UPMB13 were observed to be inferior (Figure 5).

251 While the growth of UPMB13 fluctuates in the temperature range of 25-30°C between 24 h to 72
252 h, sustained bioflocculant productions were observed, reflected by the >70% percentage flocculating
253 activities measured throughout the assay. In contrast, the flocculating activities of UPMB13 grown at 37-
254 40°C decreases with time although its growth proliferated towards the end of the test. This suggested that

255 the available bioflocculant produced might be consumed back as food substitute that induces growth and
256 thus, lowered the flocculating performances.

257 Optimum growth temperatures for *B. subtilis* were reported to be at 30-37°C with the minimum
258 temperature of 18°C and the maximum at 43°C (Korsten and Cook 1996). However, for the purpose of
259 bioflocculant production, it was concluded that the optimum temperature range for UPMB13 was 25-
260 30°C.

261 **3.7 Distribution of flocculating abilities by different culture parts**

262 Distribution of flocculating abilities by separated culture parts reflects production source and determines
263 the capacity of each part to be extracted and further purified (Liu and Cheng 2010). Naturally,
264 bioflocculants productions by microorganism can be cellularly bounded or excreted extracellularly as
265 slimes (Subramanian et al. 2009). Therefore, distribution of flocculating activities by different culture
266 parts of UPMB13 was investigated (Figure 6).

267 High flocculating activities around 89% can be observed by both the culture broth and the cell-
268 free supernatant ($p>0.05$) with visible flocs formed. In comparison, the cells' flocculating activities were
269 significantly lower at only 39.4% ($p<0.05$), with no flocs formation observed. Charge destabilizations by
270 the cation supplied may explain the percentage flocculating activities achieved by the cells apart from the
271 residual bioflocculant activities that may adhered to the cell surfaces (Wei et al. 2008).

272 The results obtained proved that the bioflocculants are extracellularly produced by UPMB13 into
273 the surrounding broth, exhibited by the cell-free supernatant similarly. Hence, the cell-free supernatant
274 was chosen as the bioflocculant source for the extraction and characterization of the bioflocculant.

275 **3.8 Genetic competence for continuous bioflocculant production**

276 UPMB13 was proven to be genetically competence for continuous production of high performing
277 bioflocculants as the ability to produce the bioflocculants persist up to its 10th generation progeny (Figure
278 7).

279 Percentage flocculating activities measured for each generation fluctuated and maintained above
280 80% ($p>0.05$) with an exception of the 10th generation, where a slight 9% decrease can be seen compared

281 to the highest measured performance at 86.9% ($p<0.05$). Visual assessment of flocs formation were also
282 positive for all generations.

283 Continuous sub-culturing had been reported to cause loss of rosy and mucoid phenotypic
284 expression of polysaccharides by the strain (Dierksen et al. 1997), whereby these characteristics are major
285 contributors to bioflocculability. Furthermore, Shih and Wu (2009) reviewed that some *B. subtilis* strains
286 have different genetic competency in EPS production. For instance, *B. subtilis subsp. (natto)* had been
287 reported to be genetically incompetent in continuous production of γ -PGA. In other reported studies
288 genetic incompetency in bioflocculant production could be overcome through ion implantation (Peirui et
289 al., 2008).

290 Hence, based on the results it was proven that UPMB13 has the genetic competence in
291 bioflocculant production even after incessant sub-culturing processes.

292 **3.9 Microscopic images of purified bioflocculant and bioflocculation**

293 From the optimum cultural conditions determined above, fermentation of 1 L batch cultures were
294 prepared to extract the bioflocculant at its best measured performance based on the kaolin assays. About
295 0.90g of purified bioflocculant can be collected from 1 L culture of UPMB13 in TSB.

296 Figure 8a shows the image of the purified bioflocculant produce by UPMB13, while Figure 8b
297 and Figure 8c display the microscopic images of the kaolin particles both before and after treated by the
298 bioflocculant, respectively. The purified bioflocculant (Figure 8a) appeared to be fibrous with smooth
299 globular structures. These are the typical attributes of γ -PGA as observed by Yang (2011).

300 The un-flocculated kaolin particles (Figure 8b) are scattered, dispersed and smaller in nature,
301 while the flocculated kaolin particles (Figure 8c) were observed to be clumped together and larger in
302 comparison. According to a review by Maximova and Dahl (2006), aggregated particles formed through
303 induced polymeric flocculations and salt coagulations can be in the form of either loose or compact
304 structures. Aggregates formed in a system with high salts concentrations with added polymeric bridging
305 are loose in nature while those formed from the low coagulant concentration with added shear during
306 bridging lead to the formation of compact aggregate structures. Therefore, referring back to Figure 8c, it
307 can be confirmed that the flocculated kaolin particles portrayed the properties of a compact aggregated

308 structures which were formed through the induced polymeric bridging of UPMB13 bioflocculant with
309 applied shear and minimal cationic aid.

310 Similar observation was also reported by Nwodo et al. (2014), whereby the bioflocculated kaolin
311 particles formed tightly weaved continuous structures with no evident spacing observed between the
312 particles. Observations of these clumped, flocculated kaolin particles proved the binding and bridging
313 treatment induced by the bioflocculant (Cosa et al. 2013).

314 **4 Conclusions**

315 Extracellular *de novo* synthesis (L-glutamic acid independent) of bioflocculants by *B. subtilis* UPMB13
316 were found to occur at an early logarithmic growth phase of 24-72 h of fermentation. Bioflocculant
317 production was found to be negatively correlated with growth at a limited supply of 100 mL of tryptic soy
318 broth media. Productions of high performing bioflocculants may be prolonged and sustained with optimal
319 fermentation conditions of sufficient nutrients provided and suitable oxygen level supplied. Proliferation
320 of bacterial growth occur at best at 25-30°C and at the optimum pH 6 while pH 7.0 was more preferable
321 for early bioflocculant productions. Bioflocculant production through submerged fermentation of *B.*
322 *subtilis* UPMB13 was proven feasible based on its genetic competency in continuous production of high
323 performance bioflocculant. The results of the research can be useful in water treatment applications.

324

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439

440 **Table 1** Growth and flocculating activities of UPMB13 at 48 h of incubation

Media	OD660	Flocculating activities (%)	Flocs presence
Tryptic soy broth (TSB)	1.49 ± 0.05	81.7	Present
TSB + L-glutamic acid (LGA)	0.04 ± 0.01	71.7	Absent
TSB + L-glutamic acid and citric acid (LGA+CA)	0.03 ± 0.01	27.7	Absent

441

442

443 **Figure captions**

444

445 **Fig. 1** The timeline of bioflocculant production by *B. subtilis* UPMB13 during growth. Bioflocculant
446 production is reflected by the flocculating activities measured

447

448 **Fig. 2** The effect of different culture media ratio on flocculating activities

449

450 **Fig. 3** The effect of various incubation shaking speed on flocculating performances of UPMB13 at 24 h
451 of incubation. Floccs were present in all treatments.

452

453 **Fig. 4** Flocculating activities and growth measured at 24 h at different initial pH. Floccs were present in all
454 treatments.

455

456 **Fig. 5** The flocculating activities of UPMB13 bioflocculant at incubation temperature range of 25-30°C (■
457) and 37-40°C (■) and the growth at 25-30°C (▲) and 37-40°C (X). Floccs were present in all treatments.

458

459 **Fig. 6** The distribution of flocculating abilities by different culture parts. The original culture is
460 represented by the broth. The broth is then centrifuged to separate both the supernatant and the cell
461 component of the culture. Floccs were absent in the cell treatment.

462

463 **Fig. 7** Flocculating activities of UPMB13 up to the 10th culture progenies. Floccs were present in all
464 treatments.

465

466 **Fig. 8** SEM images of (a) the purified bioflocculant, (b) un-flocculated kaolin particles, and (c) kaolin
467 particles flocculated with UPMB13 bioflocculant