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Adsorptive bioprocess improves yield of melanin from Pseudomonas stutzeri
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1   **TITLE:**
2   Adsorptive Bioprocess Improves Yield of Melanin from *Pseudomonas stutzeri*
3
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17

18   **SUMMARY:**
19   Here we present a protocol on adsorptive bioprocess to produce a biological product, especially
20   a pigment that inhibits its own biosynthesis and inhibits the growth of the microorganism that
21   produces it.
22

23   **ABSTRACT:**
24   Melanins are natural pigments, and the presence of indole ring and numerous functional groups
25   makes melanin an ideal choice for many applications such as UV protective agents, skincare,
26   cosmetics etc. A marine *Pseudomonas stutzeri* produces melanin without the addition of
27   tyrosine. The feedback inhibition was observed by melanin in the culture of a melanin-producing
28   marine bacterium, *Pseudomonas stutzeri*. Melanin also demonstrated microbial growth
29   inhibition. The Han-Levenspiel model-based analysis identified uncompetitive type product
30   inhibition of melanin on the cell growth. Tyrosinase enzyme, which produces melanin, was
31   inhibited by melanin. The double reciprocal plot of the enzymatic reaction in the presence of
32   different melanin concentrations revealed uncompetitive product inhibition. An adsorbent-
33   based adsorptive bioprocess was developed to reduce the feedback inhibition by melanin.
34   Different adsorbents were screened to select the best adsorbent for melanin adsorption. Dosage
35   amount and time were optimized to develop the adsorptive bioprocess, which resulted in an 8.8-
36   fold enhancement in melanin production by the marine bacteria *Pseudomonas stutzeri* (153 mg/L
37   to 1349 mg/L) without supplementation of tyrosine and yeast extract.
38

39   **INTRODUCTION:**
40   Melanins are polyphenol compounds whose monomeric unit is the indole ring. Melanins are
41   responsible for most of the black, brown, and grey colorations of plants, animals, and
42   microorganisms[1]. The most common melanins are eumelanins, which are dark brown or black
43   pigments widely distributed in vertebrates and invertebrates[2]. Cephalopods, plants, and
44   microbes are the major sources of melanin[3]. Melanin obtained from microorganisms has several

advantages over melanin from cephalopods and plants. They are fast in growth and do not cause any problems of seasonal variations. Also, they modify themselves according to the growth medium and the operating conditions provided. Considering all these facts, the microbial sources are exploited as a major source of melanin with potential commercial applications[4–6]. Guo et al. used recombinant *Streptomyces kathirae* SC-1 to produce melanin up to 28.8 g/L in an optimized medium[7]. Lagunas-Muñoz et al. Obtained about 6 g/L of melanin in recombinant *E. coli* [8]. Wang et al. obtained about 4.2 g/L of soluble melanin after medium optimization[9]. Researchers also investigated marine cultures to produce melanin. Kiran et al. produced melanin from marine *Pseudomonas* species[10]. Kumar et al. isolated *Pseudomonas stutzeri* HMGM-7 from marine seaweed, which produced melanin in nutrient broth. All the reported melanin producers require medium supplements, yeast extract and/or peptone for melanin production, which may hinder the scale-up of the bioprocess. Raman et al. investigated melanin production from *Aspergillus fumigatus* AFGRD105, which did not require costly additives[11].

A marine bacterium known as *Pseudomonas stutzeri*, isolated from seaweed, was selected for melanin production. This microorganism does not require additional supplementation of tyrosine and yeast extract for the production of soluble melanin and grows in seawater-based media, thereby reducing the potable water footprint and medium cost. Thaira et al. reported an optimized bioprocess using coconut cake meal for melanin nanopigment production using this marine bacterium in a previous publication[12]. The produced melanin was used for the highly efficient removal of heavy metals from the synthetic groundwater. However, during medium optimization, plausible feedback inhibition of melanin on the growth and product formation was identified. Therefore, in this protocol, feedback inhibition of melanin is investigated and quantified using the generalized Han-Levenspiel model. A simple and effective adsorptive bioprocess protocol is developed to reduce feedback inhibition to the maximum extent.

PROTOCOL:

1.   Growth and maintenance of *Pseudomonas stutzeri* culture.

1.1.   Obtain the bacteria *Pseudomonas stutzeri* from MTCC Chandigarh (supplied as freeze-dried ampoule).

1.1.1.   Disinfect the surface of the ampoule with alcohol (70%). Mark the ampoule near the middle of the gauge and break at the marked area.

NOTE: Care should be taken in opening the ampoule as the contents are in vacuum. Take precautions while breaking the ampoule, as a snap opening will draw the cotton plug to one end and a hasty opening will release the lyophilized fine particles of the microbe into the air.

1.1.2.   Carefully remove the cotton plug with sterilized tweezers and add 0.3 mL of Luria Bertani (LB) broth to the ampoule. Let it stand for 30 min.

88   1.1.3.   Inoculate 100 µL of the suspension to 10 mL of LB broth (in 100 mL conical flask) and
89   place it in the incubator shaker at 180 rpm maintained at 37 °C. Simultaneously transfer 100 µL
90   of the suspension to the solid LB agar and spread to obtain isolated colonies. Incubate it at 37 °C
91   for overnight.

92

93   1.2.   Once the culture has grown, store them at 4 °C.

94

95   1.3.   Do subculture after every 4 weeks by streaking a colony onto a fresh LB agar plate and
96   incubating the plate at 37 °C for overnight for the colonies to appear.

97

98   **2.   Inhibition studies of melanin on the growth and enzyme activity**

99

100   2.1.   Prepare the culture medium by adding 51.4 g/L glucose, 49.8 g/L coconut cake meal, 0.09
101   g/L, 0.03 g/L and 0.05 g/L of $CuSO_4$, $FeSO_4$ and $MgSO_4$, respectively, in artificial seawater (**Table**
102   **of Materials**). Perform all the experiments in a 250 mL shake flask with 15 mL filling volume at
103   200 rpm, 37 °C, and initial pH of 7.

104

105   2.2.   Add different concentrations of melanin (100 mg/L, 200 mg/L, 500 mg/L, and 1000 mg/L)
106   into each culture medium at the start of the bioprocess.

107

108   2.3.   Measure specific growth rate, the enzyme activity of the cells, and melanin at different
109   time intervals of the bioprocess for each added melanin concentration (section 3.2).

110

111   **3.   Modeling melanin inhibition kinetics**

112

113   3.1.   Use the following generalized equation of Han and Levenspiel[13] to model the inhibition
114   kinetics of melanin production,

115 $$\mu = \mu_{max} \cdot \left(1 - \frac{p}{p^*}\right)^n \cdot \frac{s}{\left(s + K_s \cdot \left(1 - \frac{p}{p^*}\right)^m\right)} \qquad (1)$$

116   Where,
117   μ = apparent specific growth rate (1/h)
118   μ_{max} = maximum specific growth rate (1/h)
119   p = product concentration (g/L)
120   p^*= critical product concentration at which growth ceases (g/L)
121   s = substrate concentration (g/L)
122   m,n = parameters
123   Above equation can be rewritten as
124     $\mu = \mu_{obs} \cdot \frac{s}{(s + K_{sobs})}$ (2)
125   Where,
126     $\mu_{obs} = \mu_{max} \left(1 - \frac{p}{p^*}\right)^n$ (3)
127   and $K_{obs} = K_s \cdot \left(1 - \frac{p}{p^*}\right)^m$ (4)

128 The above equation can be rearranged to a double reciprocal plot of 1/μ versus 1/s, as given
129 below

130 $$\frac{1}{\mu} = \frac{1}{\mu_{obs}} + \frac{K_{obs}}{\mu_{obs}} \cdot \frac{1}{s}$$ (5)

131

132 3.2. Draw a straight line using the data of different specific growth rates and substrate
133 concentrations using the above equation. The intercept on the Y- and the X-axis, respectively,
134 give μ_{max} and K_s, which can be substituted in eqution 3 and 4 respectively to obtain μ_{obs} and
135 K_{obs}.

136

137 Equation (3) can be written as

138 $$\ln(\mu_{obs}) = \ln(\mu_{max}) + n \cdot \ln\left(1 - \frac{p}{p^*}\right)$$ (6)

139

140 3.3. Calculate the specific growth rate of culture grown in step 2.2 and name it as μ_{obs}.

141

142 3.4. Use the value of the specific growth rate obtained in step 3.3 in equation (6). Fit the data
143 to a linear equation and calculate the slope and intercept.

144

145 3.5. Use equation (3) to calculate the maximum specific growth rate in the absence of melanin.

146

147 **4. Development of the adsorptive bioprocess**

148

149 4.1. Perform all the experiments in a 250 mL shake flask with 15 mL filling volume at 200 rpm,
150 37 °C, and initial pH of 7.

151

152 4.2. Conduct the final optimized bioprocess in a 5 L stirred tank bioreactor with a 3 L filing
153 volume. Maintain the pH of the culture between 7–7.5 and dissolved oxygen between 50%– 60%
154 of the saturation in the bioreactor studies.

155

156 **5. Selection of the best adsorbent from different available adsorbents**

157

158 5.1. Add different adsorbents such as alumina, zeolite, celite, fuller's earth, and activated
159 carbon in different flasks containing the culture broth.

160

161 5.2. Add each adsorbent separately at a concentration of 1 g/L to the culture broth and keep
162 it in a rotary shaker at 150 rpm for 12.

163

164 5.3. Separate the adsorbents by centrifugation at 5000 x g for 10 min and measure the
165 absorbance of the supernatant at 400 nm using a spectrophotometer.

166

167 5.4. Select the adsorbent which adsorbs the maximum amount of melanin from the culture
168 broth.

169

170 **6. Effect of dosage time of the adsorbent**

6.1.   Start the bioprocess and add the adsorbent yielding the highest adsorption of melanin from the culture (selected after section 5). The concentration selected at the initial step is 1 g/L.

6.2.   Measure the maximum melanin production according to the previously published protocol[12].

6.3.   Repeat step 6.1 by adding the adsorbent after 12 h of the start of the bioprocess and measure the melanin production at different times of the bioprocess.

6.4.   Repeat step 6.3 by adding the adsorbent after 24 h, 36 h, and 48 h of the start of the bioprocess.

6.5.   Compare the melanin produced in each bioprocess run and identify the best dosage time for the maximum melanin production and recovery.

7.   Effect of amount of adsorbent dosage

7.1.   Add different concentrations of the best adsorbent (0.5, 1, 2, 4, 6, 8, and 10 g/L) to the cultures at the time identified in section 6.

7.2.   Harvest the bioprocess by centrifugation at 5000 x g for 10 min.

7.3.   Collect the supernatant and calculate the amount of melanin by measuring absorbance at 400 nm.

7.4.   Wash the pellet containing adsorbent two times each with distilled water and ethanol to remove cells and other compounds attached to the adsorbent. Perform the washing step by centrifugation at 5000 x g for 10 min.

7.5.   Add a 0.1 M NaOH solution to the washed adsorbent to desorb the melanin.

7.6.   Centrifuge the mixture at 5000 x g for 10 min and measure the melanin amount in the supernatant at 400 nm by a spectrophotometer.

7.7.   Repeat the desorption process thrice or until the adsorbent is back to its normal color.

7.8.   Reduce the pH of this concentrated desorbed melanin solution to 3 or less by adding 1 N HCl using a stirrer with magnetic beads to precipitate melanin. Store the acidic solution in the refrigerator for 1 h to increase precipitation, if required.

7.9.   Wash the precipitated melanin thus obtained three times with distilled water by centrifugation at 5000 x g for 10 min, dry and store at 4 °C.

8.   Mathematical modeling of the bioprocess

8.1.   Biomass growth kinetics

8.1.1.   Quantify the biomass growth rate using the following modified Verhulset model developed for product inhibition, as described by the equation given below[14-17],

$$\frac{dx}{dt} = \mu_{obs} \cdot x \cdot \left(\frac{x_m - x}{x_m}\right)$$ (7)

Where,

x = biomass concentration (g/L)

x_m = maximum biomass concentration (g/L)

μ_{obs} = maximum specific growth rate (1/h) as given by equation (2)

Vazques et al. (2008) further derived the following equation for biomass concentration from equation (7)

$$x = \frac{x_m}{1 + exp\left[2 + \frac{4 \cdot v_x}{x_m} \cdot (\tau_x - t)\right]}$$ (8)

Where,

v_x = biomass growth rate (g/L/h) = $\mu_{obs} \cdot x$

μ_{obs} = observed specific growth rate (1/h)

τ_x = lag time (h)

t = time (h)

8.1.2.   Use Equation (8) to calculate the biomass concentration at different times of the bioprocess and simulate the growth profile of *Pseudomonas stutzeri*.

8.2.   Substrate consumption kinetics

NOTE: The substrate is used for biomass growth, product formation, and maintenance in a bioprocess.

8.2.1.   Use the following equation to balance substrate utilization in the bioprocess,

$$-\frac{ds}{dt} = \frac{1}{Y_{P/S}} \cdot \frac{dp}{dt} + \frac{1}{Y_{X/S}} \cdot \frac{dx}{dt} + m_s \cdot x$$ (9)

The majority of the glucose is used for biomass generation; therefore, for simplification, neglecting substrate consumption for product formation would reduce equation (9) to,

$$-\frac{ds}{dt} = \frac{1}{Y_{X/S}} \cdot \frac{dx}{dt} + m_s \cdot x$$ (10)

Where,

S = substrate concentration (g/L)

$Y_{P/S}$ = product yield coefficient, i.e., melanin yield coefficient (g/g)

P = product concentration (g/L), i.e., melanin concentration (g/L)

$Y_{X/S}$ = biomass yield coefficient (g/g)

m_s = maintenance coefficient (g/g/h)

256 Integrating the above equation from time t = 0 to time t = t, and substituting the value of x from
257 equation (8) gives the following equation for substrate concentration profile[17],

$$s = s_0 - \frac{1}{Y_{X/S}}[x - x_0] - \left(\frac{m_s \cdot x_m^2}{4 \cdot v_x}\right) ln \left[\frac{x_0 \cdot \left(exp^{\frac{4 \cdot v_x}{x_m}t} - 1\right) + x_m}{x_m}\right] \quad (11)$$

259 Where, s_0 = initial substrate concentration (g/L) and x_0 = initial biomass concentration (g/L)

261 8.2.2. Use equation (11) to calculate the substrate concentration at different time intervals of
262 the bioprocess or if substrate concentration is known, then use the same equation to estimate
263 the parameters given in the equation.

265 8.3.   Melanin production kinetics

267 8.3.1. Use Ludeking-Pirate equation to simulate melanin production. However, melanin is
268 growth associated and therefore, the non-growth associated product formation term is
269 neglected, and the simplified equation can be written as,

$$\frac{dp}{dt} = \alpha \cdot \frac{dx}{dt} \quad (12)$$

271 Where,
272 α = Ludeking-Pirate constant linked to growth associated product formation (g/g)
273 Integrating equation (12) from time $t = 0$ to $t = t$ result in the following equation for product
274 concentration profile

$$p = \alpha \cdot (x - x_0) \quad (13)$$

277 8.3.2. Use equation (13) to calculate α.

279 8.4.   Kinetic parameter estimation

281 8.4.1. Estimate all parameters mentioned above by the nonlinear least square method of
282 parameter estimation using any suitable software tool (e.g., Microsoft Excel solver tool).

284 8.4.2. Use batch experiment data for parameter estimation and minimize the sum of squared
285 error (SSE) using the Solver tool, as given by the following equation,
286 Minimize,

$$SSE = \sum_{i=1}^{n}\left(x_{sim} - x_{exp}\right)^2 \quad (14)$$

288 Where,
289 x_{sim} = simulated value and x_{exp} = experimental value

291 **9.   Analytical techniques**

293 9.1.   Melanin extraction

295 9.1.1. Centrifuge the growth culture at 5000 x g for 10 min to remove the biomass.

297   9.1.2. Add an equal volume of 1 M NaOH to the supernatant.

298

299   9.1.3. Acidify the alkaline supernatant to pH 2 by adding 1 N HCl to precipitate the melanin.

300

301   9.1.4. Centrifuge the acidified supernatant at 12000 x g for 20 min to collect melanin.

302

303   9.1.5. Wash the collected melanin thrice with 5 mL of distilled water, dry overnight at room
304   temperature (RT) and store at 4 °C[18].

305

306   9.2.   Melanin quantification

307

308   9.2.1. Prepare different concentrations of melanin solutions (0–500 mg/L) by dissolving the
309   appropriate amount of melanin in artificial seawater (pH 8).

310

311   9.2.2. Measure the absorbance at 400 nm using spectrophotometer[18].

312

313   9.2.3. Prepare calibration curve using above data.

314

315   9.2.4. Centrifuge the growth culture at 5000 x g for 10 min to remove the biomass and measure
316   the absorbance of the supernatant at 400 nm. Calculate the melanin concentration in the growth
317   culture using the calibration curve prepared in step 9.2.3

318

319   9.3.   Tyrosinase assay

320

321   9.3.1. Follow the protocol given below for the tyrosinase enzyme assay developed by Ren et al.
322   (2013)[19] to determine the enzyme activity of tyrosinase extracted from *Pseudomonas stutzeri*
323   culture broth.

324

325   9.3.2. Centrifuge the bacterial culture at 5,000 x g to collect the cells.

326

327   9.3.3. Sonicate the cells in cold 100 mM phosphate buffer of pH 6.5 and centrifuge the
328   homogenate at 9000 x g for 15 min and collect the supernatant, a source of tyrosinase enzyme.

329

330   9.3.4. Add 0.2 mL of the enzyme solution obtained in the above step to 2.8 mL of 0.1 M sodium
331   phosphate buffer (pH 6.5) containing 1 mM L-tyrosine (substrate).

332

333   9.3.5. Observe the formation of dopachrome by measuring the absorbance at 475 nm.

334

335   9.3.6. Calculate the tyrosinase activity as follows. One international unit (IU) of tyrosinase
336   activity is defined as the amount of enzyme required to oxidize 1 μmol of L-tyrosine to
337   dopachrome per minute. Calculate this by using the molar extinction coefficient of dopachrome
338   (3600 L·M^{-1}·cm^{-1}) by the following equation,

339

340 $$\frac{U}{mL} = \frac{\left(\frac{Absorbance}{minute}\right)\cdot\left(Assay\ volume(mL)\right).(dilution factor).(1000)}{(3600\ L\ .\ M^{-1}.cm^{-1}).(1\ cm).\left(enzyme\ volume\ (mL)\right)}$$ (15)

341

342 9.4.   Estimation of biomass

343

344 9.4.1. Collect 5 mL of the samples at an interval of 4 h in the Biosafety cabinet, maintaining
345 sterility. Flame the cotton plug of the flask before and after taking the samples to reduce the
346 chances of contamination. Measure the optical density (OD) of the samples at 660 nm for all
347 these samples.

348

349 NOTE: The uninoculated media serves as the blank. These readings will be used to study the
350 growth of the microorganisms.

351

352 9.4.2. Centrifuge the culture broth at 5000 x g for 10 min and 4 °C, collect the cell pellet and dry
353 the pellet for 8 h at 60 °C in a hot air oven to obtain the dry cell mass.

354

355 9.4.3. Prepare calibration curve using the OD measured in step 9.4.1 and the corresponding
356 biomass obtained in step 9.4.2

357

358 9.4.4. Use the calibration curve (OD = 0.82⬚Biomass (g/L)) to measure the unknown quantity of
359 biomass by measuring the optical density of the culture broth at any given time.

360

361 9.5.   Estimation of DNA

362

363 9.5.1. Estimate the amount of DNA in the sample by measuring the optical density at 260 nm.

364

365 9.5.2. Calculate the DNA using the following formula,

366 $$Total\ DNA\ (\mu g) = \frac{(A_{260}).\left(50\ \frac{\mu g}{mL}\right).(dilution\ factor).(0.1\ mL)}{1}$$ (16)

367 Where,
368 A_{260} = Absorbance at 260 nm
369 0.1 = Volume of the sample
370 1 = Absorbance of DNA standard solution of concentration 50 µg/mL, at 260 nm

371

372 9.6.   Estimation of protein

373

374 9.6.1. Estimate the amount of protein in the samples by using a Bradford protein estimation kit
375 (Table of Materials).

376

377 9.6.2. Add 5 mL of the Bradford reagent to 0.1 mL of the supernatant and keep it for 5 min. The
378 proteins present in the supernatant reacts with the Bradford reagent to form a colored complex.

379

380 9.6.3. Measure the absorbance at 595 nm[20] using the spectrophotometer.

381

382   9.7.   Estimation of total reducing sugar (TRS)

383

384   9.7.1.   Measure the TRS by dinitrosalicylic (DNS) method[21].

385

386   9.7.2.   Prepare the DNS solution by dissolving 1 g of DNS in 50 mL of distilled water. Add 30 g of
387   sodium potassium tartarate to this solution in small amounts till the color of the solution
388   becomes milky yellow, followed by transparent orange-yellow with the addition of 20 mL of 2 N
389   NaOH. Bring the volume up to 100 mL using distilled water and store it in the dark.

390

391   9.7.3.   Prepare glucose working solution (0.01 M) by dissolving 180 mg of glucose in 100 mL of
392   distilled water in a standard flask.

393

394   9.7.4.   Pipette out standard Glucose solution (0.2, 0.4, 0.6, 0.8 and 1 mL) into 5 separate test
395   tubes. Use a test tube containing distilled water(1mL) as a blank solution.

396

397   9.7.5.   Bring the volume up to 2 mL in each test tube by using distilled water.

398

399   9.7.6.   Add 1 mL of DNS reagent to each test tube and cover with aluminum foil.

400

401   9.7.7.   Heat the test tubes in a boiling water bath for 5 min and cool to RT.

402

403   9.7.8.   Add 9 mL of distilled water to each test tube and mix well. Measure the OD of the content
404   at 575 nm using a spectrophotometer.

405

406   9.7.9.   Measure the concentration using a calibration chart prepared with the amount of glucose
407   on the X-axis vs. absorbance at 575 nm (A_{575}) on the Y-axis.

408

409   9.8.   Particle size analysis

410

411   9.8.1.   Disperse the biosynthesized melanin in distilled water (1 mL) using a sonicator.

412

413   9.8.2.   Add the dispersed sample solution (1 mL) into the nanoparticle analyzer and measure the
414   particle size per the manufacturer's protocol.

415

416   9.9.   Transmission Electron Microscopy (TEM) analysis

417

418   9.9.1.   Dry the melanin powder obtained after extraction by keeping it at 60 °C for 8 h.

419

420   9.9.2.   Load the melanin powder in the TEM sample matrix and measure the size using the
421   Transmission Electron Microscope following the operator protocol.

422

423   **REPRESENTATIVE RESULTS:**
424   **Figure 1A**,**B** demonstrates growth and melanin production under the influence of different initial
425   melanin concentrations. The cell growth and melanin production decreased substantially with

426   the increasing initial concentration of melanin. At 500 mg/L initial melanin concentration in the
427   medium, the biomass and melanin reduced to almost 50% of the maximum value obtained in
428   control. At 1000 mg/L of initial melanin concentration, there was marginal melanin production.
429   The biomass growth rate reduced significantly at 1000 mg/L initial melanin concentration. **Figure**
430   **1C** shows intracellular tyrosinase activity in culture broth at different initial melanin
431   concentrations. As mentioned in the figure, the tyrosinase activity decreased by a factor of 4 in
432   the presence of 1000 mg/L initial melanin concentration in the broth compared to the control.
433   These results clearly show that the higher melanin concentration in the culture adversely affects
434   the tyrosinase enzyme activity and cell growth.
435
436   It is necessary to identify the type of inhibition so that a solution can be obtained accordingly.
437   Therefore, the feedback inhibition of the melanin on the tyrosinase enzyme was identified using
438   the Han-Levenspiel model (protocol section 3.1, equation (3)). **Figure 1D** quantifies the effect of
439   melanin inhibition on the maximum specific growth rate of microorganisms. The maximum
440   specific growth rate of *P. stutzeri* in the optimized medium decreased from about 0.25 h^{-1} to
441   about 0.01 h^{-1} . The line drawn in the figure 1D is explained by the following equation,

442    $$\mu_{obs} = 0.25 \left(1 - \frac{p}{p^*}\right)^{0.91} \text{ for optimized medium.} \tag{17}$$

443
444   The values of coefficients m and n are greater than zero, confirming uncompetitive inhibition by
445   melanin. The critical inhibitory concentration of melanin was about 1050 mg/L. The Lineweaver-
446   Burke plot (**Figure 2A**) obtained from the tyrosinase activity also confirmed the uncompetitive
447   inhibition of melanin onto crude tyrosinase. The values of Michelies constant and the maximum
448   enzyme velocity changed at different inhibitor concentrations, as shown in **Figure 2A**.
449
450   The above steps quantified the melanin inhibition onto the growth of the microorganisms and
451   inhibition of its production. Therefore, it was necessary to remove melanin from the broth to
452   reduce feedback inhibition and increase the productivity of the bioprocess. The present protocol
453   describes the development of this adsorptive bioprocess. Different commercially viable and
454   easily available adsorbents such as zeolite, celite, alumina and activated carbon were used to
455   study their melanin adsorption potential from the broth. The method used to identify the
456   potential adsorbent is explained in protocol sections 4 and 5. As **Figure 2B** indicates, alumina was
457   the best adsorbent, which adsorbed almost 60% of the melanin produced from the broth at pH
458   8. Melanin is ionized in the anionic form in the basic pH of the broth and hence solubilizes in
459   water. Further, it was necessary to determine the adsorption capacity of alumina for melanin.
460   Adsorption isotherms describe the adsorption capacity of a given adsorbent. Melanin adsorption
461   onto alumina was better explained by Langmuir isotherm, and therefore, this isotherm was used
462   to find the binding capacity of alumina. Langmuir adsorption isotherm assumes monolayer
463   adsorption, non-interaction between the adsorbed ions, the uniform energy of adsorption in the
464   plane of the adsorbent surface and availability of the identical binding sites for monolayer
465   adsorption. The linear form of Langmuir adsorption isotherm can be written as follows [22]:
466
467    $$\frac{C_e}{q_e} = \left(\frac{1}{q_{max}}\right) \cdot \left(\frac{1}{b}\right) + \frac{C_e}{q_{max}} \tag{18}$$

468

469   Where, Ce (mg/L) is the equilibrium concentration of melanin in the solution, $qmax$ is the
470   maximum adsorption capacity, which is the amount of adsorbate required to form a monolayer,
471   qe (mg/g) is the amount of melanin adsorbed per specific amount of adsorbent at equilibrium
472   and b is a constant, which represents the affinity of adsorbent to adsorbate.

473

474   **Figure 2C** shows experimental values fitted to the Langmuir adsorption isotherm equation (18).
475   The maximum adsorption capacity of melanin onto alumina from pure melanin solution and the
476   broth at pH 8 was 127.4 mg melanin/g of alumina and 113.8 mg melanin/g of alumina,
477   respectively. The amount of proteins, sugars, and cells/DNA adsorbed onto alumina was
478   negligible (>2 mg/g) and therefore is not reported.

479

480   **Figure 2D** shows the effect of dosage time of alumina on melanin production. Dosage time of 12
481   h demonstrated better melanin yield. At this stage, cells were in the exponential growth phase
482   with a biomass concentration of 1.7 g/L. The pH of the medium was 7.3 and the melanin
483   concentration was about 130 ± 15 mg/L. There was about a 50% increase in melanin production
484   when adsorbent was added to the culture after 12 h than the normal culture grown in the
485   optimized medium. The effect of adsorbent concentration on melanin production was
486   investigated in the next step of adsorptive bioprocess development.

487

488   **Figure 3A** shows that melanin production increased as the adsorbent dosage increased.
489   Tyrosinase enzyme activity also improved by increasing the adsorbent dose, which is evident
490   from the figure. At 10 g/L adsorbent concentration, the reducing sugars were completely
491   consumed by *P. stutzeri* by 48 h. After identifying suitable alumina dosage time and amount, an
492   adsorptive bioprocess was carried out in a 5-L stirred tank bioreactor with the addition of alumina
493   (10 g/L) at the time when melanin concentration reached about 130 mg/L. The melanin
494   production increased to about 1349 mg/L (**Figure 3B**), which is about an 8.8-fold increase as
495   compared to the batch process in the unoptimized medium (**Figure 3C**). The solid lines in **Figure**
496   **3B** represent simulation results of the adsorptive bioprocess. **Table 1** compares the growth and
497   product formation parameters for a normal batch bioprocess and adsorptive bioprocess. The
498   table is self-explanatory and proves that adsorptive bioprocess increased the specific growth
499   rate, yield coefficients and hence productivity of melanin biosynthesis. Further, **Figure 3C**
500   compares the melanin production at different time intervals in a normal batch bioprocess and an
501   adsorptive bioprocess in a stirred tank bioreactor under optimized conditions. It also shows the
502   amount of melanin remaining in the supernatant during the adsorptive bioprocess. The melanin
503   concentration remained nearly constant in the supernatant, in the range of 95–110 mg/L in the
504   presence of alumina, thus reducing the inhibitory effect. From the figure, it is evident that there
505   is an enhancement in melanin production due to the addition of adsorbents in a batch process.
506   **Figure 3D** demonstrates the adsorption of melanin onto alumina in the adsorptive bioprocess.
507   The alumina adsorbed the melanin produced in the broth. The growth medium containing free
508   melanin and melanin bound on alumina particles was collected at the end of the bioprocess. A
509   0.1 M NaOH solution was added to the alumina particles obtained to desorb melanin from
510   alumina particles, which is exhibited in **Figure 3D**. Particle size analysis of the dispersed solution
511   of biosynthesized melanin in water shows that particles are nanoparticles with a size of 32 nm ±

512   0.98 nm (**Figure 4A**). **Figure 4B** shows an aggregate of melanin molecules of size in the nano
513   range. Thus, particle size analysis and transmission electron microscopy show that the melanin
514   molecules are of nano size.
515
516   **FIGURE AND TABLE LEGENDS:**
517   **Figure 1: Profiling of melanin with respect to growth.** (**A**) The growth of the cells. (**B**) The melanin
518   production at different melanin concentrations. (**C**) Decrease in the enzyme activity with an
519   increase in the inhibitor concentration. (**D**) Maximum specific growth rate at different melanin
520   concentrations in the optimized medium.
521
522   **Figure 2: Adsorption profile of melanin**. (**A**) The Lineweaver-Burke plot for tyrosinase enzyme
523   from Pseudomonas Stutzeri. (**B**) adsorption of melanin by different adsorbents and their zeta
524   potential values. (**C**) Langmuir adsorption isotherm for adsorption from melanin solution. (**D**)
525   Effect of time of adsorbent dosage on melanin production.
526
527   **Figure 3: Bioprocess sketch and melanin adsorption**. (**A**) Effect of adsorbent (alumina) on
528   melanin production and enzyme activity of cells. (**B**) Melanin production in stirred tank bioreactor
529   with the addition of adsorbent alumina. (— simulation results). (**C**) Production of melanin in
530   normal batch bioprocess and adsorptive bioprocess. (**D**) Images of melanin adsorption onto
531   alumina and its desorption
532
533   **Figure 4: Particle size and TEM analysis.** (**A**) Particle size analysis of the biosynthesized melanin.
534   (**B**) An aggregate of melanin particles seen in TEM.
535
536   **Table 1: Growth parameters.** Values of growth parameters obtained through equations (8), (13)
537   and (17) are the values of v_x, α and μ_{max}, respectively. $Y_{X/S}$ is calculated from grams of biomass
538   produced per gram of carbon substrate consumed, and $Y_{P/S}$ is calculated from grams of product
539   formed per gram of carbon substrate consumed. The last column shows a percentage increase in
540   values of growth parameters of adsorptive bioprocess with respect to the normal batch process.
541
542   **DISCUSSION:**
543   Product inhibition is a major bottleneck in bioprocessing which leads to reduced productivity.
544   Several methods exist to reduce product inhibition, such as continuous bioprocess and *in situ*
545   product removal techniques. However, these options require a complete overhaul of the existing
546   bioprocessing facility[23–28]. Adsorptive bioprocess does not require a significant overhaul in the
547   existing facility and can be implemented very easily. The melanin exhibited feedback inhibition
548   on tyrosinase as well as reduced the growth of the *Pseudomonas stutzeri* culture. The critical
549   concentration of melanin to completely cease the growth was about 1050 mg/L. The extracellular
550   melanin isolated from *Schizophyllum commune* showed significant antifungal activity against
551   dermatophytic fungi, *Trichophyton simii*, and *Trichophyton rubrum* and antibacterial activity
552   against *Escherichia coli, Proteus sp.*[29]. Melanin produced by *Auricularia auricula* displayed
553   inhibitory activity on three bacterial strains, *Pseudomonas aeruginosa* PAO1, *Pseudomonas*
554   *fluorescens* P-3 and *E. coli* K-12, and there was a significant reduction in biomass with the increase
555   in pigment concentration[30]. Melanin produced by *Streptomyces lusitanus* DMZ-3 showed

556   cytotoxic activity on brine shrimps[31]. Although microbial melanin inhibition is reported in the
557   literature, the finding that melanin also inhibits the source organism itself and exhibits feedback
558   inhibition is not reported to date. Therefore, an adsorptive bioprocess was developed as given in
559   the protocol to reduce/nullify melanin inhibition. Alumina was selected as the best adsorbent for
560   melanin. Zeolite and fuller's earth are usually cation exchangers, due to which they did not bind
561   large amounts of melanin. Celite and activated carbon are good physical adsorbents; however,
562   they adsorb non-polar compounds.
563
564   On the contrary, alumina is a polar adsorbent and hence was able to adsorb polar and anionic
565   melanin from the fermentation broth. The maximum adsorption using alumina coincided with
566   the maximum difference in zeta potential (**Figure 2B**), confirming the suitability of alumina as the
567   adsorbent for melanin in basic pH. Therefore, alumina was chosen for the development of
568   adsorptive bioprocess. The dosage time was optimized to 12 h after the start of the bioprocess
569   (**Figure 2D**). The melanin production decreased when alumina was added just after the
570   inoculation. Alumina is known to partially inhibit microbial growth. Since melanin was not
571   biosynthesized in the medium just after the inoculation, free alumina might have inhibited the
572   growth and hence production of melanin. Alumina addition at 36 hours after inoculation also did
573   not show improvement in melanin production. By this time, melanin was already produced in
574   quantities, which would have initiated inhibitory effects. These findings indicate that time of
575   addition of adsorbent is crucial for successful adsorptive bioprocess, especially if the adsorbent
576   is inhibiting the microbial growth. The adsorptive bioprocess using alumina as an adsorbent
577   reduced melanin inhibition. The adsorptive bioprocess in a 5 L bioreactor increased melanin
578   production from 520 mg/L to 1349 mg/L, using the same optimized medium. Thus, adsorptive
579   bioprocess further increases the productivity by 2.6 times and 8.8 times as compared to an
580   optimized batch process and an unoptimized process, respectively. Yield coefficient of melanin
581   over substrate increased from 8.23 mg/g to 22.5 mg/g by increasing adsorbent dosage.
582   Moreover, the total sugars were not detectable in the culture broth at the end of the bioprocess
583   at an adsorbent dosage of 10 g/L, thus reducing the loading on the effluent treatment plant. The
584   process described here can be easily incorporated to reduce/nullify product inhibition in any bio-
585   production facility without further modification in the existing plant, thus providing operational
586   flexibility.
587

592
593   **DISCLOSURES:**
594   The authors do not have any conflict of interest
595

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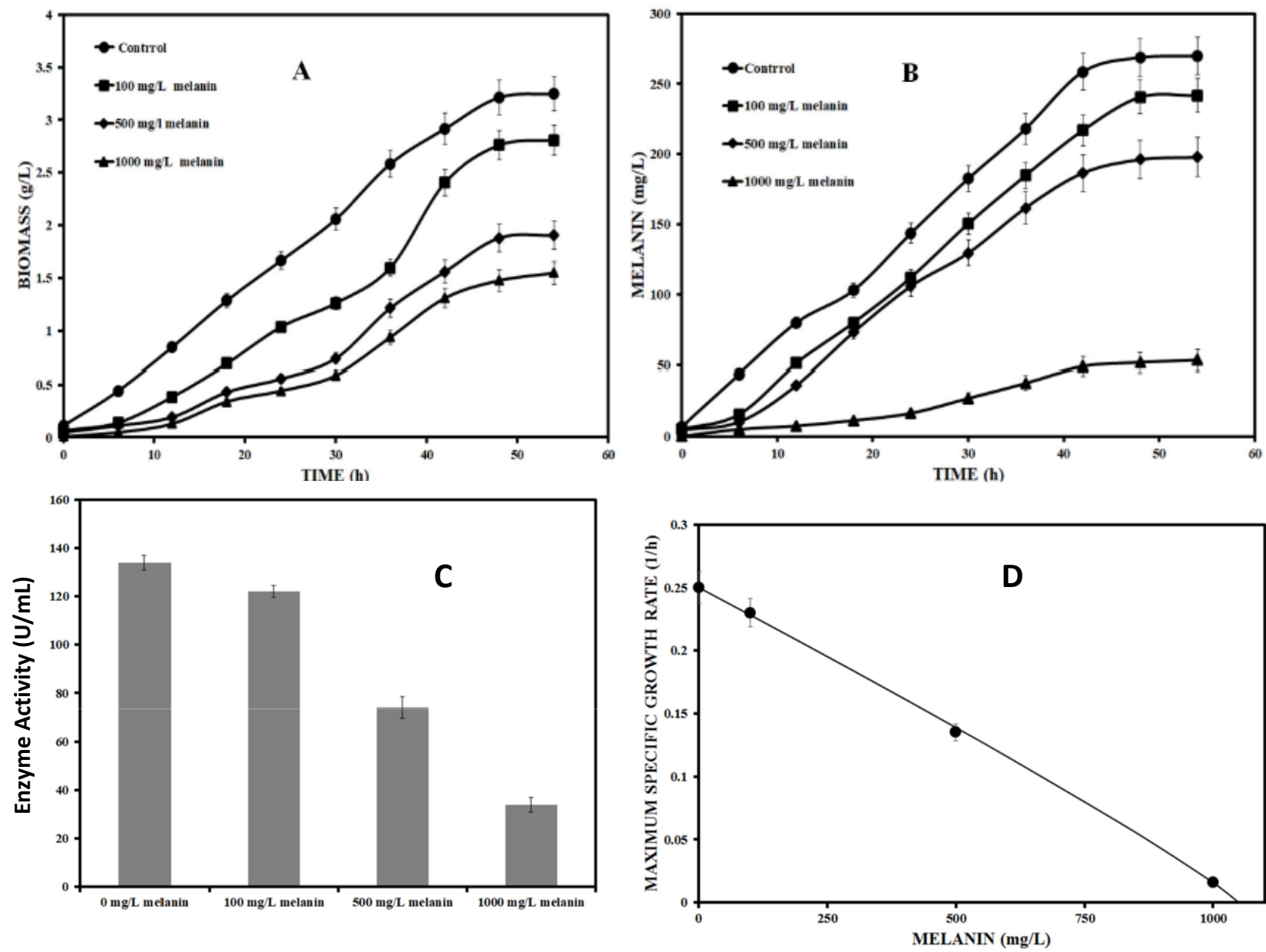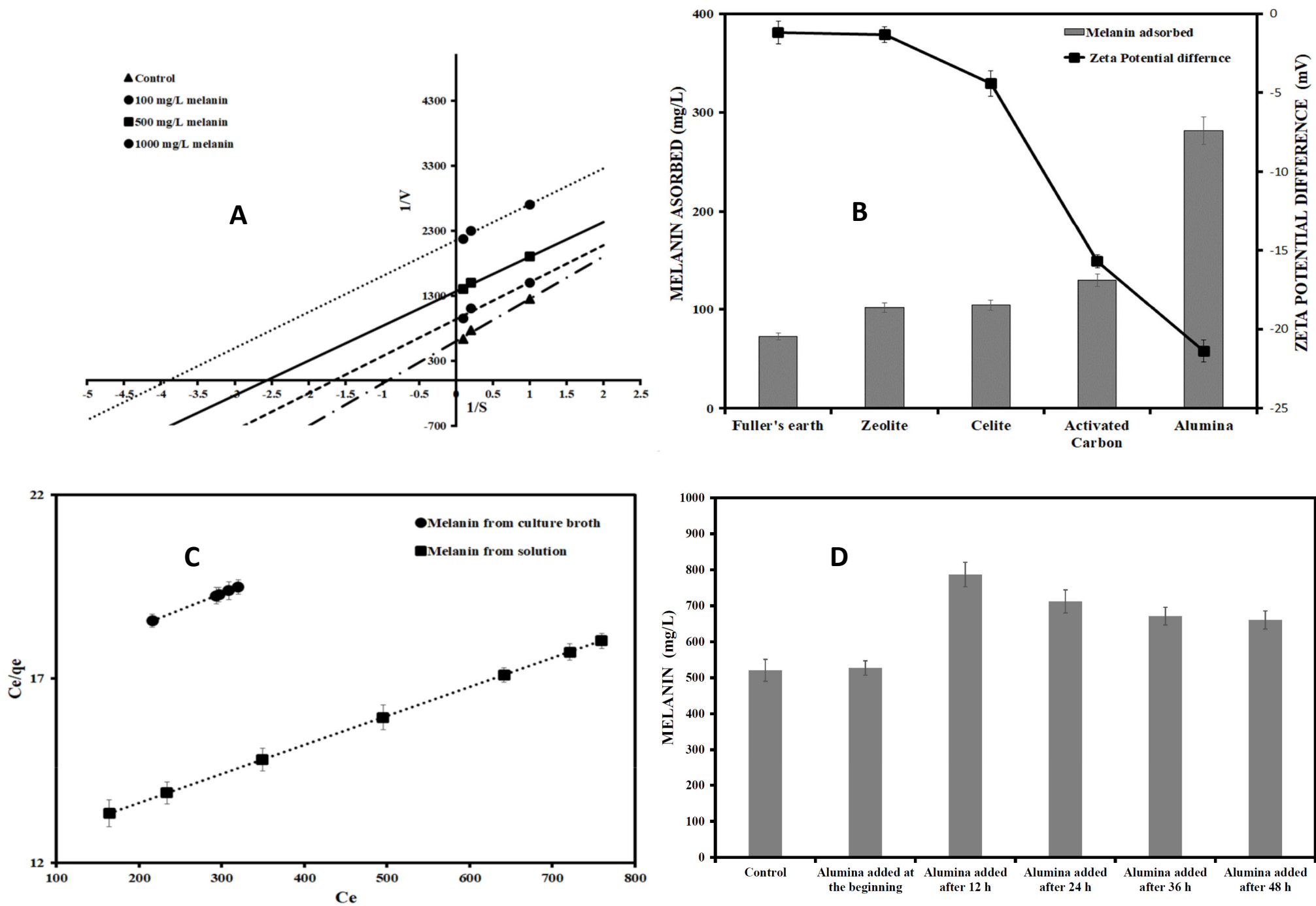678   DMZ-3c. *BioMed Research International*. **2014**, 1–11 (2014).
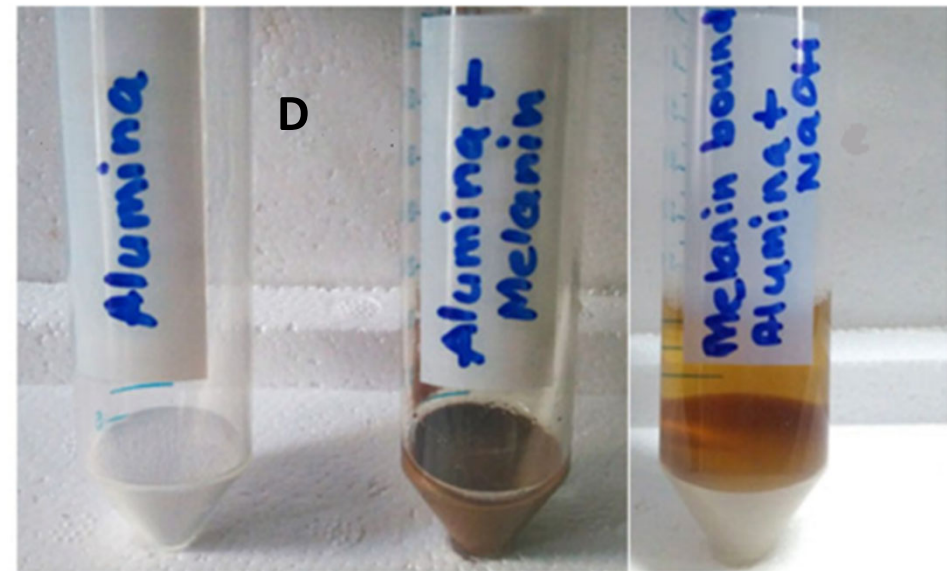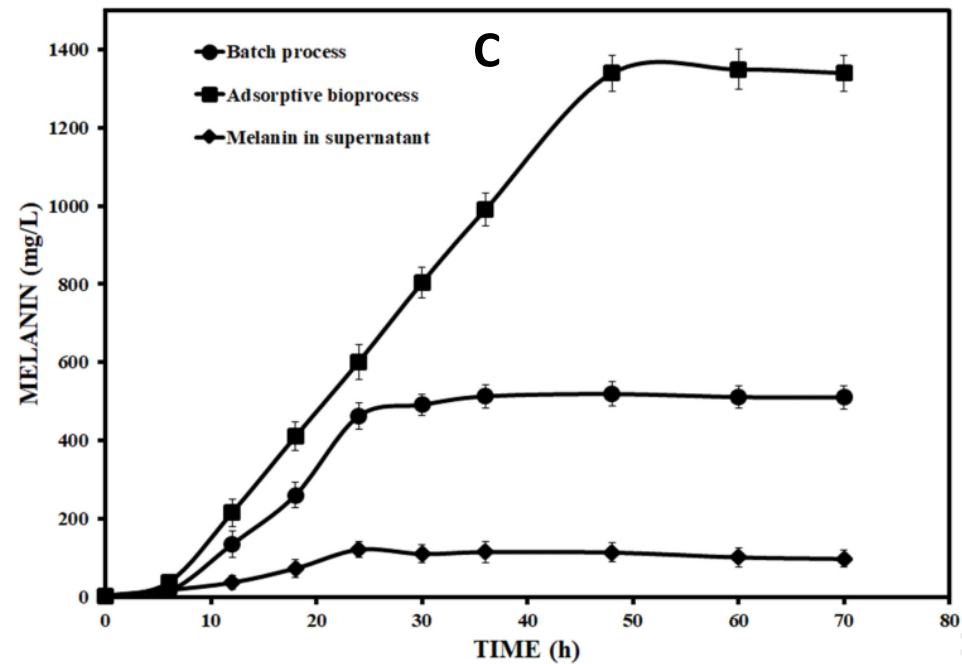
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680

Figure 1

Figure 2

Figure 3

Figure 4

Parameter	Normal batch process	Adsorptive bioprocess	% increase wrt batch process
μ_{max} (1/h)	0.145	0.25	72.4
v_x (g/l/h)	0.15	0.28	86.6
α (g/g)	0.081	0.105	29.6
$Y_{X/S}$ (g/g)	0.14	0.21	50
$Y_{P/S}$ (mg/g)	8.3	22.5	177
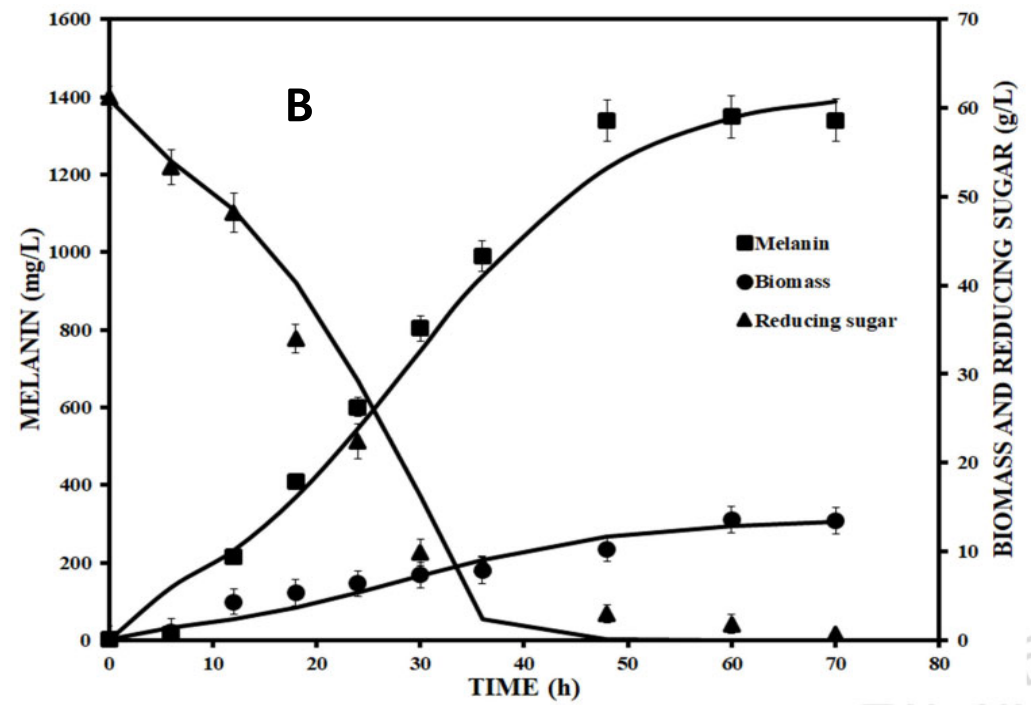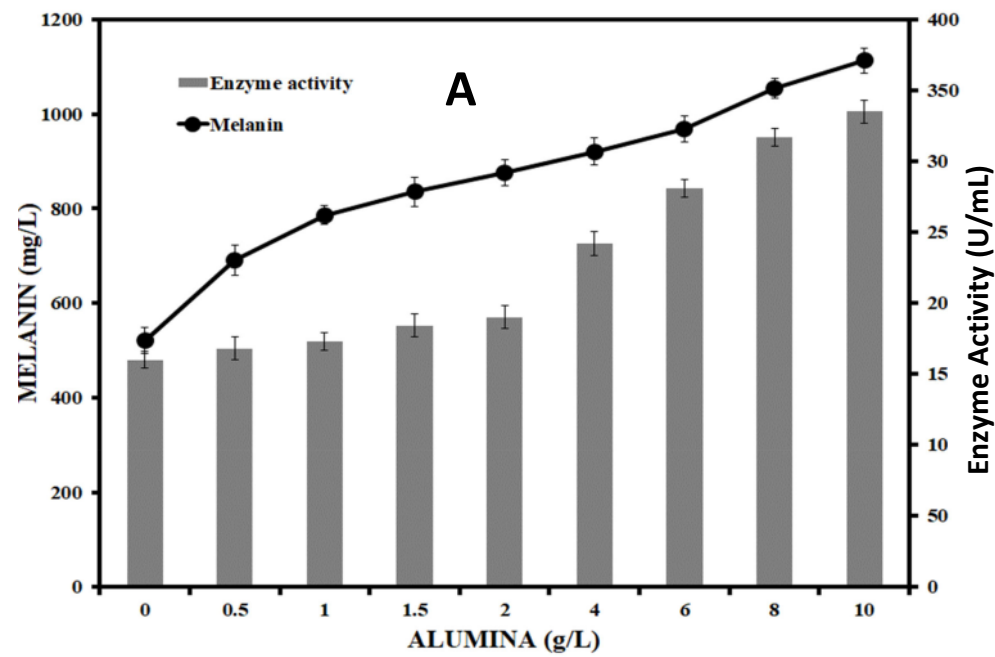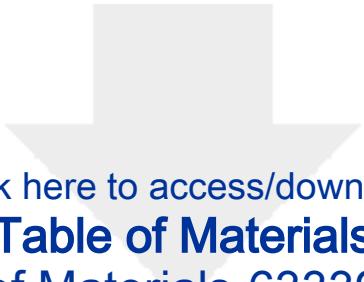
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Table of Materials
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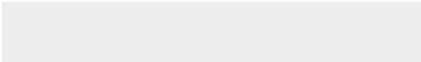
Dear Editors,

Thank you very much for reviewing the manuscript. We changed the manuscript as per the queries raised by you. Following are our replies to the queries raised.

Kindly note that all the changes have been made and are in the "TRACK CHANGES" mode in the word file

Query 1: Please note that the section numbers and step numbers have been formatted to fit the Journal standard. Please review.
Reply: As suggested by the editors, the manuscript was reviewed for the formatting changes.

Query 2: Howe is this done? Please add more details regarding retrieving and maintenance of cultures.
Reply: As suggested by the editors, relevant details have been added for the retrieval and maintenance of the cultures.

Query 3: How is subculturing done. Please add the steps for subculturing. A citation would suffice.
Reply: As suggested by the editors, the methodology for the subculturing has been added .

Query 4: Artificial seawater? Is this prepared? Any specific composition?
Reply: The artificial seawater broth was purchased from Himedia , Bangalore, India. The specifications have been added in the text.

Query 5: If the details for each parameter mentioned in this step is detailed below, please reference the section numbers of the specific methods in this step?
Reply: The details of the section has been added in the text.

Query 6: The sentence s incomplete. Please revise.
Reply: The suggested modifications have been incorporated.

Query 7: Revised. Please check.
Reply: The corrections were made in the revised sentence.

Query 8: How?
Reply: The reference used for melanin estimation has been incorporated in the test.

 Query 9: How is the bioprocess harvested?
Reply: The sentence has been modified to mention the harvesting step.

 Query 10: How is the absorbent washed. Is any centrifugation or vortexing involved? Any specific volume of water and ethanol used? Any specific time duration for each washing step?
Reply: The centrifugation details required for washing have been incorporated in the text.

Query 11: What is added to reduce the pH? Please specify the added constituent and the volume added to achieve the mentioned pH.
Reply: The details of the HCL used for the acidification have been added. The volume of the HCl will depend on the melanin yield, hence the endpoint pH has been mentioned.

Query 12: Any centrifugation or voretxing involved? What is the time duration of each wash?
Reply: The details of the centrifugation have been incorporated in the manuscript.

Query 13: Is this stimulate or simulate?
Reply: The conditions were simulated for computer modeling.

Query 14: Please cite the reference number

Reply: The citation has been added in the text.

Query 15: How is the culture collected? How is the sterility ensured? What is the volume collected each time to measure the OD?
Reply: The details of the query raised has been addressed and incorporated in the manuscript.

Query 16: After how long of culturing is the culture centrifuged?
Reply: The samples were collected at an interval of 4h. This is mentioned in the preceding step.

Query 17: Sample? Please specify.
Reply: It is a commercial DNA sample and has been mentioned in the manuscript.

Query 18: What is the dilution?
Reply: The dilution factor has been included in the text.

Query 19: Is this commercial or prepared in the lab? If its prepared, please include a citation. In case it is purchased, ensure that it is included in the Table of Materials.
Reply: The estimation was done using the commercial kit from Himedia and has been mentioned in the manuscript.

Query 20: Volume?
Reply: The volume utilized has been added in the text.

Query 21: What is the volume of water used? How much of melanin is dispersed?
Reply: The volume has been included in the text.

Query 22: Volume?
Reply: The volume has been included in the text.

Query 23: Is this performed according to the manufacturer's protocol? Please cite.
Reply: The changes have been incorporated as suggested.

Query 24: How? Using an oven or dry at RT? For how long is the melanin dried?
Reply: The melanin is kept in the oven for 8h for drying. The conditions have been included.

Query 25: How is the sample loaded to the TEM sample matrix
Reply: The protocol as specified by the manufacturer was followed. This has been mentioned.

Query 26: Please check if the section number is correct.
Reply: The section no. as suggested has been corrected.

Query 27: Is this correct?
Reply: The notation of the units have been changed from 1/h to h^{-1}

Query 27: Please check if the section numbers are correct.
Reply: The section number mentioned are correct.

Query 28: Please include a title for each figure.
Reply: The title has been included as suggested.

Query 29: Please include a brief description for the Table
Reply: The changes as suggested have been incorporated in the main text.

Query 30: As we are a methods journal, please ensure that the Discussion explicitly covers the following in detail in 3-6 paragraphs with citations: a) Critical steps within the protocol, b) Any

modifications and troubleshooting of the technique, c) Any limitations of the technique, d) The significance with respect to existing methods, e) Any future applications of the technique.
Reply: The text explicitly speaks about the suggested points.