Chapter 10

Immobilization of *Rubia tinctorum* L. Suspension Cultures and Biomass Production

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Abstract

Plants are natural sources of valuable secondary metabolites used as pharmaceuticals, agrochemicals, flavors, fragrances, colors, biopesticides, and food additives. There is an increasing demand to obtain these metabolites through more productive plant tissue applications and cell culture methods due to the importance of secondary metabolites.

Immobilization of plant cells is a method used in plant cell cultures to induce secondary metabolite production. In this method, plant cells are fixed in or on a supporting material or matrix such as agar, agarose, calcium alginate, glass, or polyurethane foam. In the present study, three natural lignocellulosic materials, loofah sponge, and the long fibers of sisal and jute, were used to immobilize suspended *R. tinctorum* cells.

Key words Immobilization, Plant cells, Suspension culture, Biomass, Lignocellulosic matrices, Loofah, Sisal, Jute, Secondary metabolite production

1 Introduction

The immobilization of plant cells is one of the secondary metabolite-inducing techniques used in cell cultures of many plant species. This technique comprises the fixation of cells onto a solid support, a membrane or into a solid matrix to increase the stability of the cells (Fig. 1). Most reports have indicated that immobilized cultures may induce the yields of secondary metabolite production 16 times more in comparison to cell suspension cultures. Due to the secretion of metabolites to the culture medium, the cells are not only disturbed but also may carry on secondary metabolite production [1, 2].

Plant cells are often in the form of aggregates; they both cling to each other and surfaces as well; therefore, agitation is needed for plant cell culturing. On the other hand, agitation is detrimental to plant cells because of their low tolerance to shear stress. Furthermore, cell-to-cell contact is another important

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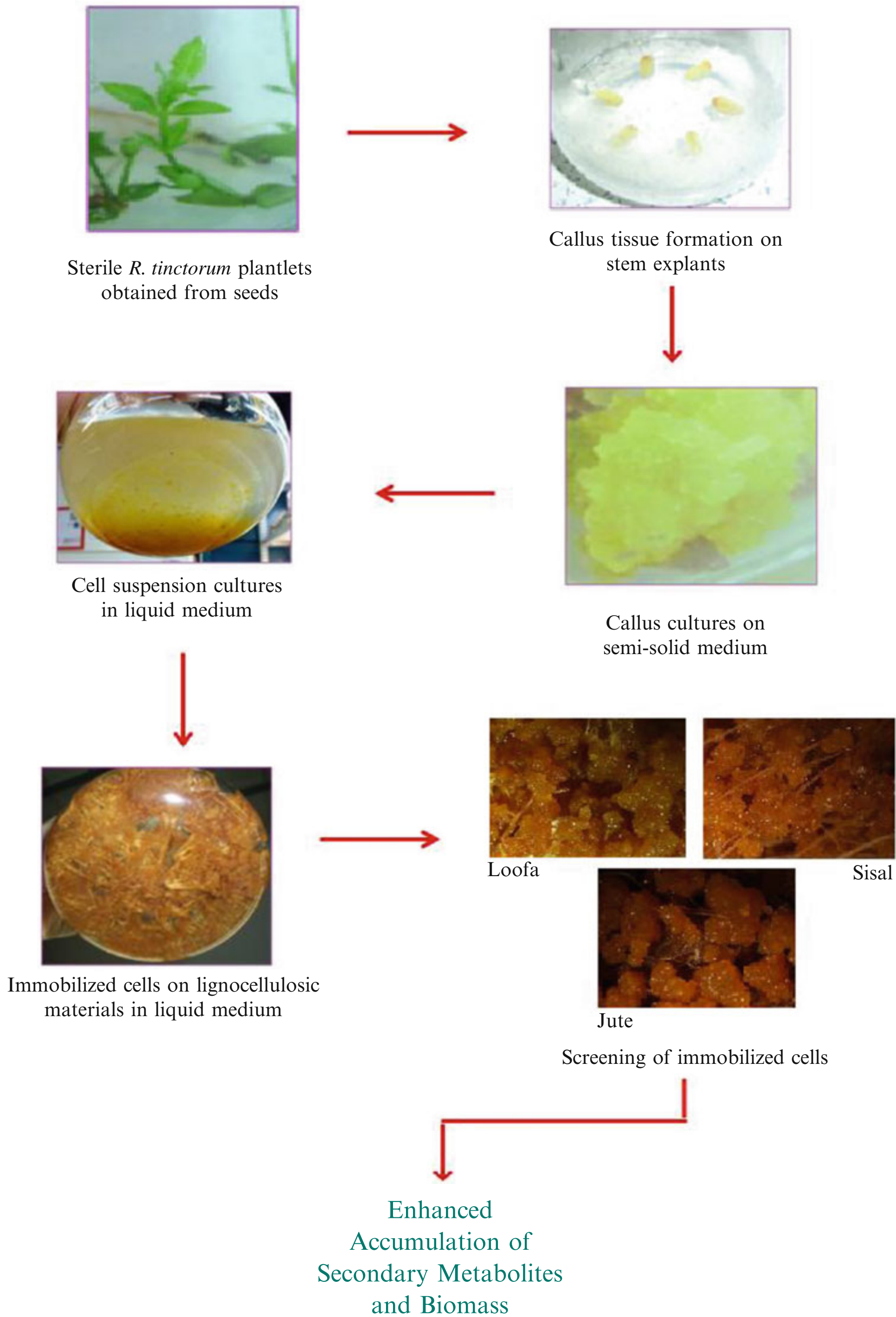

Fig. 1 Steps of immobilization procedure from plantlets to immobilized cells and enhanced accumulation of secondary metabolites and biomass

point in cell metabolism which allows metabolites to move from one cell to another. Secondary metabolite production is usually higher in slow growing or non-growing cultures [3, 4]. The immobilization of plant cells addresses all of these factors and may be the best approach for increasing secondary metabolite production. This technique offers the potential for dealing with the limitations of secondary metabolite production in liquid plant cell cultures.

In order to immobilize cells, ideally grown suspension cultures at exponential phase must be obtained firstly. The suspension cultures are started from callus or protoplast cultures of the plant species of interest (Fig. 1).

Although there is no ideal immobilization matrix for plant cell cultures, cell carriers should have some features in order to ease the production of biomass and secondary metabolites. The most common matrices used for plant cell immobilization are calcium alginate [1] and reticulate polyurethane foam [2]. Besides these matrices, agar and agarose [5], polyester [6] glass [7] fibers, hybrid sol–gel matrices [7], loofah sponge, jute and sisal fibers [8] have also been utilized for immobilization.

We used lignocellulosic materials (loofah, sisal, and jute) in order to immobilize suspended cells of *Rubia tinctorum* L. (Fig. 2). Immobilizing cells from *R. tinctorum* L. on lignocellulosic materials provided around three times higher biomass production in comparison to suspension cultures. Alizarin and purpurin contents of immobilized cells were 6 and 23 times higher in inoculated cells, respectively. In this chapter, the methods for establishing an ideally grown suspension culture and immobilization of suspended cells on lignocellulosic materials are discussed.

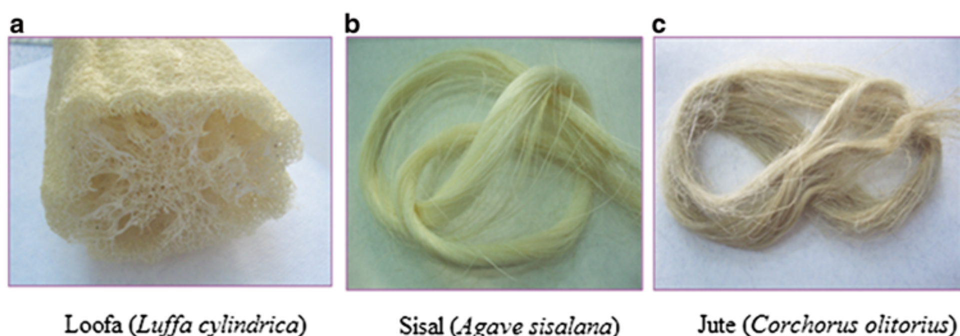

Fig. 2 Natural lignocellulosic materials used for immobilization of plant cell cultures

2 Materials

2.1 Laboratory Materials and Equipment

1. Laminar flow cabinets.
2. Bunsen burner.
3. Dissecting forceps and scalpel.
4. Beakers.
5. Cylinders.
6. pH-meter.
7. Analytical balance.
8. Stirrer with hot plate.
9. Culture vessels containing semisolid media.
10. 250 ml Erlenmeyer flasks for use as culture vessels for liquid medium.
11. 250 or 500 ml bottles for storage of liquid cultures.
12. Orbital shaker.
13. A digital camera fitted on a compact stereomicroscope.
14. Autoclave.
15. Incubator at 170 °C.
16. Autoclavable 30 mesh sieve.
17. Filter paper.
18. Sterile petri dishes to weigh callus on an analytical balance in laminar flow cabinet (sterile aluminum foil can also be used instead of petri dishes).
19. Funnel.
20. Distilled water sterilized by autoclaving (121 °C for 30 min at a pressure of 1.2 kg/cm^2).

2.2 Plant Material

1. Internode parts of shoots, which were grown from the shoot tips of clonally micropropagated plantlets in micropropagation medium (Table 1).

2.3 Immobilization Materials

1. Loofah (mature and dried fibrous fruit of *Luffa cylindrica*) (Fig. 2a).
2. Sisal (fibers of *Agave sisalana* leaves) (Fig. 2b).
3. Jute (fibers of *Corchorus olitorius* leaves) (Fig. 2c).

2.4 Stock Solutions, Semisolid and Liquid Media

1. Stock solutions (1 mg/100 ml) of 2,4-dichlorophenoxy acetic acid (2,4-D), indole-3-butyric acid (IBA), and kinetin and store in the freezer at 4 °C.
2. Stock solutions of Murashige and Skoog (MS) medium [9] (Table 2).

Table 1
Contents of micropropagation, callus initiation (CI), and liquid CI (L-CI) media

Medium name	Media composition				
	Basal medium	Growth regulators	Sucrose	Agar	pH
Micropropagation medium	MS	0.5 mg/l IBA	3 %	0.7 %	5.8
Callus initiation medium (CI)	MS	0.1 mg/l 2,4-D 0.5 mg/l BA 0.5 mg/l Kinetin	3 %	0.7 %	5.8
Liquid CI medium (L-CI)	MS	0.1 mg/l 2,4-D 0.5 mg/l BA 0.5 mg/l Kinetin	3 %	–	5.8

Table 2
Composition of MS medium

MS medium[a]			
Stock solutions	g/l	g/l for 100 ml stock solution[b]	Stock code
KI	0.00083	0.0166	MS1
$MgSO_4 \cdot 7H_2O$	0.37	7.4	MS2
$MnSO_4 \cdot 4H_2O$	0.0223	0.446	
$ZnSO_4 \cdot 7H_2O$	0.0086	0.172	
$CuSO_4 \cdot 5H_2O$	2.5E-05	0.0005	
KH_2PO_4	0.17	3.4	MS3
$CaCl_2 \cdot 2H_2O$	0.44	8.8	MS4
H_3BO_3	0.0062	0.124	MS5
$Na_2MoO_4 \cdot 2H_2O$	0.00025	0.005	
$CoCl_2 \cdot 6H_2O$	2.5E-05	0.0005	
$Na_2 \cdot EDTA$	0.0373	0.746	MS6
$FeSO_4 \cdot 7H_2O$	0.0278	0.556	
Nicotinic acid	0.0005	0.01	MS7
Pyridoxine·HCl	0.0005	0.01	
Thiamine·HCl	0.0001	0.002	
Glycine	0.002	0.04	MS8
NH_4NO_3	1.65	No stock solution	–
KNO_3	1.9	No stock solution	–

[a]Dissolve all the stock solutions, NH_4NO_3, and KNO_3 in enough deionized water and make it up to 1 L. Adjust the pH to 5.8 (if necessary, add agar at this step) and autoclave for 15 min at 121 °C at a pressure of 1.2 kg/cm
[b]Use 5 ml stock solution for 1 L medium

3. *Micropropagation medium (semisolid MS medium)*: MS components, 0.5 mg/l IBA, 3 % sucrose, 0.7 % agar, pH 5.8 (Table 1).
4. *Callus initiation medium (CI) (semisolid MS medium)*: MS components, 0.1 mg/l 2,4-D, 0.5 mg/l BA, 0.5 mg/l kinetin, 3 % sucrose, 0.7 % agar, pH 5.8 (Table 1).
5. *Liquid CI medium (L-CI)*: MS components, 0.1 mg/l 2,4-D, 0.5 mg/l BA, 0.5 mg/l kinetin, 3 % sucrose, pH 5.8 (Table 1) (*see* **Notes 1** and **2**).

2.5 HPLC Analyses of Samples

1. Ultrasonic device.
2. SpeedVac concentrator.
3. Thermo Hypersil C-18 100 μm × 2.1 μm × 3 μm column.
4. HPLC-grade trifluoroacetic acid (TFA).
5. HPLC-grade acetonitrile.
6. HPLC-grade methanol.
7. HPLC-grade alizarin and purpurin (reference compounds).
8. HPLC-Grade HCl.
9. HPLC-Grade Na_2CO_3.
10. HPLC-grade ethyl acetate.

3 Methods

3.1 Stability Tests of Immobilization Materials

1. Place pieces (approximately 2 g DW) of each immobilization material (loofah sponge and fibers of sisal and jute) into glass beaker (*see* **Note 3**).
2. In the first stability test, autoclave samples three times for 15 min at 121 °C at 1.2 kg/cm^2. There should be no change in physical characteristics after the last autoclaving (*see* **Note 4**).
3. In the second stability test, put samples into incubator and subject them at 170 °C for 60 min three times. Avoid any change in physical characteristics except a little browning after the last incubation (*see* **Note 4**).

3.2 Preparation of Immobilization Materials

1. Cut loofah sponge transversely in 1 cm sections (approximately 2 g DW). Place two grams (DW) of sisal and jute fibers and loofah sponge into 250 ml flasks such that they fill the bottom area (*see* **Note 3**).
2. Wash fibers under tap water for 15 min. Soak them in boiling water for 30 min and wash them again three times with 200 ml distilled water. Close flask's mouth with cotton and cover it with aluminum foil (*see* **Note 5**).

3. After the washing process, place fibers into an incubator for drying and sterilization for 60 min at 170 °C. Soak them with sterile distilled water (approximately 5–10 ml) before addition of suspended cells (*see* **Note 6**).

3.3 Screening of Immobilized Cells on Natural Lignocellulose Materials

1. Use a digital camera fitted on a compact stereomicroscope and take images of immobilized cells on immobilization material.

3.4 Immobilization of *R. tinctorum* L. Cell Suspension Cultures

1. Cut internodes of shoots grown from the shoot tips of clonally micropropagated plantlets, in 1 cm sections and place them into CI medium (Fig. 1). Incubate them at 26 ± 1 °C in the darkness (*see* **Note 7**).
2. After 4 weeks, subculture calli grown on internode explants to fresh CI medium for producing more callus biomass. Incubate them at 26 ± 1 °C in the darkness (*see* **Note 8**).
3. Subculture callus cultures at least four times at 2-week interval to obtain friable callus. This step will facilitate in obtaining suspended cells in the liquid medium (*see* **Note 9**).
4. At the end of the fourth week of callus culturing, weigh 2 g (fw) callus for each flask and inoculate into 50 ml L-CI medium in 250 ml flasks in laminar flow cabinet under sterile conditions (*see* **Note 10**).
5. Four weeks later, filter liquid medium with cell clumps and suspended cells through a 30 mesh sieve. Dilute 25 ml filtrate (consisting of only suspended cells, not clumps) (8×10^5 living cells/ml) to 50 ml with L-CI medium and subculture four times at 14-day interval (*see* **Note 11**).
6. Adjust 25 ml of these suspension cultures to 50 ml, 75 ml, and 100 ml with L-CI medium (inoculation ratio 1/2, 1/3, and 1/4, respectively) and pour onto the sterilized immobilization materials in 250 ml flasks with two replicates (Fig. 3). Cultivate immobilized cells on an orbital shaker at 100 rpm and 26 ± 1 °C in the darkness (*see* **Note 12**).

3.5 Determination of Fresh and Dry Weights and Relative Dry Weight Ratios

1. At the fourth week of the immobilized cells culturing, filter the biomass through a funnel and normal filter paper. Remove cell clumps from the immobilization materials and measure the total fresh weights (g) of biomass for each flask (*see* **Note 13**).
2. After freeze-drying, calculate dry weights (g) and relative dry weight ratios (dry weight/fresh weight) (*see* **Note 14**).

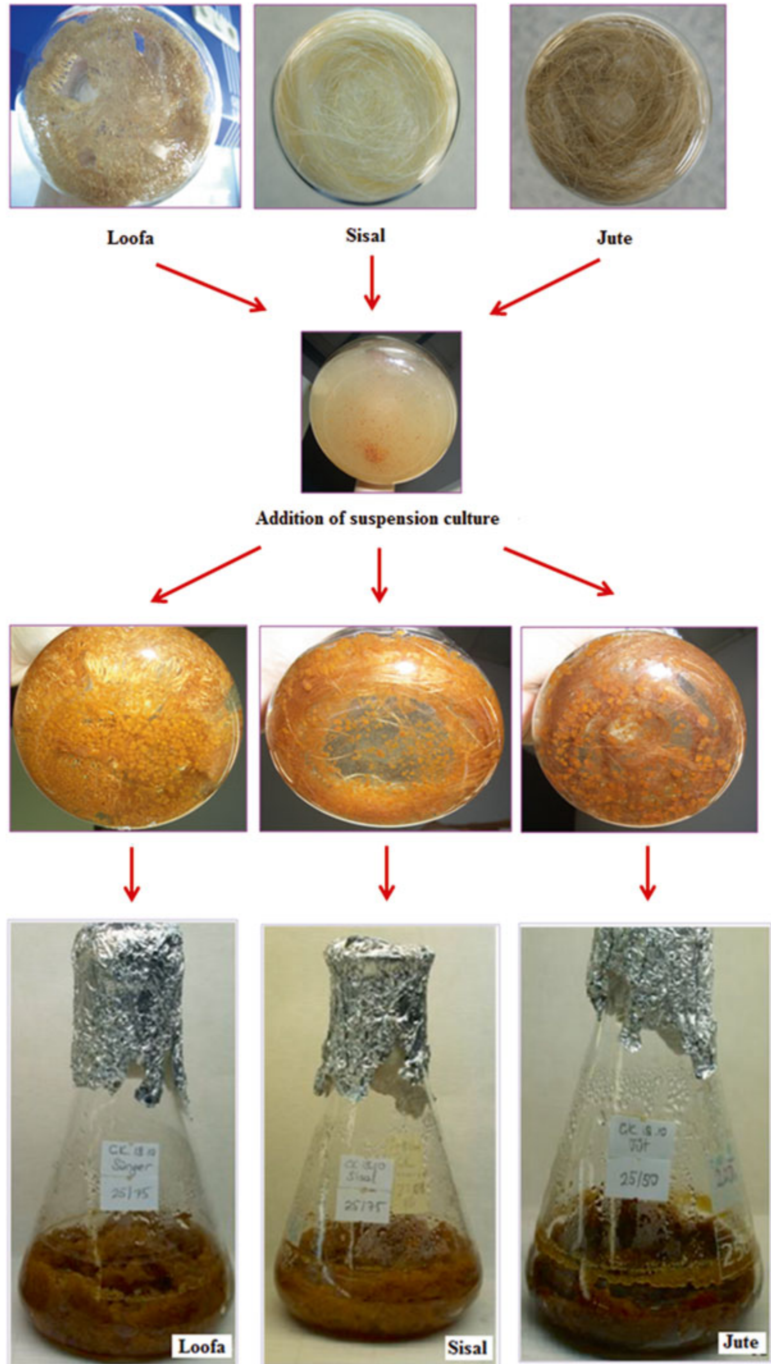

Fig. 3 Immobilization of *Rubia tinctorum* L. suspension cultures on lignocellulosic materials

3.6 HPLC Analysis of Samples

1. Extract the samples three times for 10 min with 5 ml methanol. Filter each extract, combine and evaporate them to dryness under vacuum at 60 °C (*see* **Note 15**).
2. Hydrolyse the residues by refluxing with 10 ml 5 % HCl for 1 h. Neutralize the samples with 1 M Na_2CO_3 and extract them four times with 10 ml ethyl acetate. Combine each extract and evaporate them to dryness under vacuum at 60 °C (*see* **Note 16**).
3. Dissolve all samples with 10 ml methanol and analyze them with Thermo Hypersil C-18 100 μm × 2.1 μm × 3 μm column. The mobile phase should be 0.01 % TFA in water (solvent A) and acetonitrile (solvent B) with gradient elution of 27 % B for 2.38 min; from 27 % B to 50 % B in 4.83 min; to 55 % B in 2.46 min; hold at 95 % B for 1.2 min and finally return to initial condition (27 % B) for 1.2 min respectively (*see* **Note 16**).
4. The flow rate during analysis should be 750 μl/min and inject 5 μl for each sample. The detector monitors the fluent at 250 nm.
5. Establish calibration curves by dissolving 10 mg reference compounds (alizarin and purpurin) with 100 ml HPLC grade methanol. Construct the calibration curves by plotting the peak areas of alizarin and purpurin versus their concentrations (*see* **Note 17**).

4 Notes

1. Nutrient medium should be autoclaved for 15 min at 121 °C at a pressure of 1.2 kg/cm^2 and then should be stored in darkness at last for 1 month. Liquid media should be shacked before use.
2. Adjust pH of the media to 5.8 with 0.5 M NaOH and 0.5 M HCl. Instead of NaOH, solutions like KOH can be used to adjust pH. If the volume of the medium is low (e.g., 100–200 ml), try to use solutions at lower concentrations (0.1–0.5 M).
3. Immobilization material should cover bottom of the flask as much as possible to obtain homogeneity in cultures.
4. Stability tests should be done prior to study. Once the tests are performed, there is no need to repeat them again before every experiment.
5. Among the other factors, cotton and aluminum foil are the most important factors to keep the culture sterile during culture period. Use tight cotton plug in flask without any space. Aluminum foil should cover the neck of flask completely (Fig. 3).

6. It is very important to soak immobilization materials with 5–10 ml distilled water before addition of suspended cells. In failing to do so, they will absorb nutrient medium and the volume of culture medium will be decreased.
7. Explants can be cut smaller than 1 cm up to 2 mm. If the desired metabolite is synthesized in light, then the cell culture should be incubated in light conditions.
8. Subculturing period is a tentative parameter for callus cultures. For fast growing callus, the subculture period should be shorter. In contrast, some callus types grow more rapidly and need to be subcultured more frequently (e.g., every 7–10 days).
9. In order to enhance the quality of cell cultures, subculture callus tissues at least four times before transferring to the liquid medium. The number of subcultures of friable callus can be reduced.
10. Biomass amount for inoculation should be optimized prior to the study. Optimum callus biomass amount for inoculation varies depending on the plant species. Some callus types adapt to liquid medium much easier than the others. It is very important to shorten the lag phase. If the culture enters to log phase quickly, this means the culture is in a good condition and the cells have started to divide. Callus biomass amount for inoculation can be reduced to 0.5 g/50 ml liquid medium in these cases. However, in some cases, 2 g callus is not enough to initiate cell culture otherwise up to 3–4 g callus is used to initiate suspension cultures. Conditioned medium can be a solution in these situations. The liquid medium in which cells are grown can be diluted (1/10 or 1/5) with fresh medium and can be used as a conditioned medium. This application will help to shorten the lag phase.
11. Use sieves with different mesh sizes (30–100) for obtaining suspended cells. If sieve mesh size is smaller, suspension culture will have smaller suspended cells/aggregates. However, cell suspension will contain lower number of cells per ml that would take longer lag phase. Therefore, sieve mesh size is amongst the most important factors for determining the quality of cell suspension culture and should be optimized prior to the study.
12. Reduce cell inoculums of fast growing cells to 1/10. Lag phase duration can also be used as a parameter here.
13. Before weighing, wash biomass with 50 ml distilled water to remove culture medium leftovers at the filtering step. Fresh and dry weights of filter papers should also be determined prior to filtration.

14. Freeze-drying is not necessary to determine dry weight. Incubation in an oven at 50–60 °C or air-drying can also be used. However, if the desired metabolite is unstable, freeze-drying is the most suitable method.
15. Powder the samples before extraction.
16. Use HPLC grade chemicals and solutions.
17. Prepare calibration curves by serially diluting the stock solutions of alizarin and purpurin twofold with methanol (100 mg/ml; 50 mg/ml; 25 mg/ml; 12.5 mg/ml; 6.25 mg/ml; 3.125 mg/ml).

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