RESEARCH ARTICLE

Toward enhanced hyperforin production in St. John's wort root cultures

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During the past decades, several trials targeted a stable, sustainable and economic production of St. John's wort (*Hypericum perforatum*) extract. The value of this extract stems from its use to treat depression and skin irritation due to its hyperforin content. Previously, hyperforin-forming *in vitro* root cultures were established. Here, detailed growth and production kinetics have been analyzed over 40 days of cultivation. In the first 10 days, sucrose was completely hydrolyzed to glucose and fructose. The ammonium consumption supported the increase in the biomass and hyperforin production. When sucrose was replaced with glucose/fructose, the linear growth phase started 6 days earlier and resulted in a higher space-time-yield. The maximum hyperforin production was $0.82 \text{ mg L}^{-1} \text{ day}^{-1}$, which was 67 % higher than in the sucrose-supplemented standard cultivation. Buffering the sucrose-supplemented medium with phosphate caused a 2.7-fold increase in the product to biomass yield coefficient. However, the combination of monosaccharides and buffering conditions did not cause an appreciable improvements in the production performance of the shake flask approaches. A potential scalability from flask to lab-scale stirred bioreactors has been demonstrated. The results obtained offer a basis for a scalable production of hyperforin and a sustainable source for a tissue culture-based phytomedicine.

KEYWORDS

hyperforin, *Hypericum perforatum*, production kinetics, root cultures, sucrose

1 | INTRODUCTION

The 'local to global' transition of phytomedicines started with a sparse number of people using their simple tools for collecting the plants in the wild, which was the debut of the self-made phyto-complex in folk medicine. Dramatic changes in the strategies of plant production starting from cultivation up to marketing usually skyrocket within years when a good safety index with potent treatment is confirmed [1]. A similar scene is applied to *Hypericum perforatum* (Figure 1A, St.

John's wort [SJW]). It is a valuable medicinal plant, which has been used as a healer for more than 2000 years. Since its first prescriptions in 400 BC by the Greek physicians Galen, Dioscorides, Pliny, and Hippocrates [2,3], it became one of the most valuable sources for the best-seller phytomedicine worldwide. The bulk of the SJW phyto-complex is encompassed by a broad spectrum of secondary metabolites, mainly naphthodianthrone (hypericins) and polyprenylated polycyclic acylphloroglucinols (hyperforins; Figure 1D). However, its antidepressant and skin healing properties are mainly due to its hyperforin content [4–7]. Translucent oil glands are the storage organelle for hyperforin but hypericin is housed in dark nodules distributed in leaves and flowers (Figure 1C, D). Hypericin-containing preparations

Abbreviations: DO, dissolved oxygen; g_X , growth factor; r_X , biomass formation rate; SJW, St. John's wort; STBR, stirred tank bioreactor.

possess photosensitizing capacity leading to phototoxicity after extended solar irradiation [8]. Thus, the formulation of lipophilic supercritical CO₂-based extracts (38.8% hyperforin-rich but hypericin-free) in ointments and oils for topical application is desired [9]. However, a sustainable and high quality source of SJW plants is still challenging due to the climatic fluctuations, variable soil properties, plant diseases, and genotypic variation [10,11]. Beside the environmental factors, the post-harvest processes are also proved to cause batch-to-batch variability [12–14]. Thus, in vitro cultivation of SJW offers a promising biotechnological tool for long-term supply of stable elite clones [15,16]. Despite the reported plethora of trials for in vitro tissue and organ cultures, systems with hyperforin forming properties are scarce [17,18]. Previously, SJW in vitro-grown root cultures were successfully established in our laboratory [19]. Lipophilic extracts prepared from these cultures are hyperforin-rich, in contrast to those obtained from roots of field-grown plants (Figure 1B). Interestingly, these in vitro roots overcome the traditional wild-plant limitation of cost/efficacy ratio for production of extracts with high medicinal value. First, because they are devoid of hypericin, which diminishes the need of costly CO₂-based supercritical extraction; second, they are free from chlorophyll, whose presence is known to interfere with the downstream processing [19,20]. Recently, pure hyperforin has been proven to have anti-proliferative and anti-migratory effects in 2D and 3D artificial skin constructs of human dermal fibroblasts [6]. In this context, the downstream processing of the hyperforin-rich petroleum ether extract and its use as a potential anti-proliferative agent using human keratinocytes, fibroblasts as well artificial skin constructs were investigated in detail [7,20].

Agrobacterium rhizogenes-mediated transformation of SJW plants results in hairy roots. Different from the above mentioned auxin-induced adventitious roots, hairy roots are fast growing cultures independent of growth regulators. Nevertheless, the hyperforin content of these hairy root cultures is still an open question [21–25]. In addition, the genetic modification in these cultures discourages its use as a source for phytomedicines in many countries.

For providing a sustainable and economically feasible source for a biotechnological production of high quality phyto-extracts, the scalability of hyperforin-forming auxin-induced root cultures replacing shake flasks with lab scale stirred aerated/pneumatic bioreactors plays the key role. At the commercial level, the scalability of *Panax ginseng* in vitro root cultures with average production of 45 tons fresh weight/year (CBN Biotech, South Korea) [26] is an excellent example for a successful up-scaling in the pharma-industry. Similarly, Cui et al. [27] conducted a successful 500 L airlift bioreactor-based cultivation of hypericin-producing SJW root cultures. However, the authors did not address the formation of hyperforin in these roots.

PRACTICAL APPLICATION

The article tests the concept of the cultivation of root cultures derived from *Hypericum perforatum* for the biotechnological production of the pharmaceutical drug hyperforin. The study offers an alternative technique for a sustainable supply of hyperforin-rich extracts and overcomes the environmental obstacles that cause batch-to-batch variability via establishing an in vitro cultivation system. In addition, it addresses possible options to manipulate the cultivation process toward enhanced product yield in shorter cultivation time. This attempt may open new avenues to support a stable quality of St. John's wort extracts for commercial purposes.

In the current study, a rational bioprocess design describing the aforementioned formation of hyperforin in the hypericin-free root cultures is investigated. Previously, it was observed that hyperforin formation in these cultures was growth associated [19], offering the possibility of tailoring the growth medium toward enhanced biomass growth. For proper understanding of the underlying effects, changes in the medium composition during the cultivation time were explored, properly monitored and correlated to the hyperforin production kinetics.

2 | MATERIALS AND METHODS

2.1 | Chemicals

The MS medium for the cultivation of root cultures was purchased from Duchefa (Haarlem, The Netherlands). Indole-3-butyric acid (IBA) was from Acros Organics, Geel, Belgium. Formic acid, glucose, fructose, and all salts were from Carl Roth (Karlsruhe, Germany). Sucrose was from Nordzucker AG (Braunschweig, Germany). Methanol HPLC grade and petroleum ether were ordered from Fischer Chemicals (Zürich, Switzerland). Acetonitrile and acetone were obtained from VWR Chemicals (Darmstadt, Germany). Salt-free hyperforin was ordered from Cayman Chemical (Biomol, Hamburg, Germany).

2.2 | Maintenance of adventitious root cultures at flask scale

Root cultures were maintained at 250 mL shake flask level according to the previously established system by Gaid et al. [19]. The obtained root cultures were grown in the dark at $25 \pm 2^\circ\text{C}$ and 70 min^{-1} (50 mm orbital diameter). Every 40 days, 0.5 g of the young whitish root filaments

FIGURE 1 (A) *Hypericum perforatum* entire plant. (B) Root system of field-grown plant. (C) Leaf with translucent glands spread over the lamina (hyperforin-rich; black arrow) and marginal dark nodules (hypericin-rich; red arrow). (D) Formulae of hypericin which accumulates in the dark nodules and hyperforin which is housed in the translucent glands

were transferred to a shake flask containing 100 mL of standard 1/2 MS medium [28] supplemented with 1 mg L⁻¹ IBA. Unless stated otherwise, the standard medium was supplemented with 15 g L⁻¹ sucrose. In order to determine the biomass growth kinetics, two biological repeats were prepared (20 flasks per each repeat). During the cultivation time (40 days), the root biomass was harvested every second day and the fresh weight was recorded after washing away the remaining medium with distilled water followed by stripping the surface moisture through pressing the biomass against filter paper. Dry weight was recorded after confirming a stable weight during freeze drying at -20°C in a Beta 2-8LSCbasic lyophilizer (Christ, Harz, Germany). Per flask, 10 mL of the medium was stored for further record of the hydrogen ion (pH), sugar and ammonium concentrations as well as electrical conductivity as discussed later. The biomass was stored at -20°C in a sealed nitrogen gas-flushed plastic bag in the dark to measure the hyperforin content, as described later.

2.3 | Bioreactor cultivation

Bioreactor-based cultivations were performed in a 3 L lab-scale stirred tank bioreactor (STBR) (Applikon, Schiedam, The Netherlands). To study the growth kinetics, four STBRs were started in parallel after inoculation with 5 g fresh biomass per 1 L medium (7.5 g/1.5 L medium) supplemented with 15 g L⁻¹ sucrose. A similar set of bioreactors was used to test the effect of monosaccharides via replacement of sucrose with a mixture of glucose (8 g L⁻¹) and fructose (7 g L⁻¹). Each STBR was supported with Rushton turbines (6-bladed impeller rotates at 50 min⁻¹). The cultivation period was terminated at various time intervals (10, 20, 30, and 40 days, one STBR at a time). The dry and fresh weights were determined followed by determination of the hyperforin content as discussed in Sections 2.2 and 2.8, respectively. In all STBRs, the aeration was supported with an aeration tube embedded in the medium. The stirrer movement supported the homogeneity of cultures during the cultivation and the oxygen input

was ensured via direct air inflow through the aeration tube. The airflow rate was adjusted at 0.15 L min^{-1} (according to 0.1 vvm) during the whole cultivation period. The supplemented air was as atmospheric air composition. Similar to shake flask cultivation, STBRs were kept in the dark via wrapping the STBR in aluminum foil. The temperature was kept at $25 \pm 2^\circ\text{C}$. Peripheral elements were composed of pH (Type 405-DPAS-SC-K8S/225, Mettler Toledo, Columbus, USA), and dissolved oxygen (DO) (VisiFerm DO ESC 225 HO, Hamilton Germany GmbH, Höchst, Germany) electrodes, temperature probe, sampling and gassing tubes, cold trap, and exhaust tube. The cold trap was brought to 15°C via cryostat. Sampling of the medium was identical for all four STBRs (10 mL every 2 days) and was carried out via a three-way stopcock. Medium was used to investigate the sugar concentration and pH. At the end of the cultivation period, the whole biomass was harvested and prepared as mentioned for the shake flask cultivations. The fresh and dry weights were measured followed by determination of the hyperforin content. Online data acquisition of temperature, DO, and pH were processed during the cultivation period via Hamilton sensors (Solaris Biotech solutions, Mantova, Italy).

2.4 | Bioprocess kinetics

To describe the biomass growth during the whole cultivation, the volumetric biomass formation rate (r_X) and the growth factor (g_X) were defined according to Eqs. (1) and (2), respectively [29].

$$r_X = \frac{X_t - X_0}{t} \quad (1)$$

$$g_X = \frac{X_t - X_0}{X_0} \quad (2)$$

$X_t - X_0$ is the difference between the fresh biomass (g L^{-1}) at cultivation time t (X_t) and X_0 , the biomass inoculating concentration at the beginning of the cultivation time. The specific growth rate μ is of particular interest since it is applicable to various kinetic models (Eq. (3))

$$\mu = \frac{1}{X_t} \cdot \frac{X_t - X_0}{t} \quad (3)$$

Apart from r_X , g_X , and μ , yield coefficients are important parameters, which explain the phenomenological growth, describing different relationships [30]. The biomass to substrate yield coefficient ($Y_{X/S}$), the product to substrate yield coefficient ($Y_{P/S}$), and the product to biomass yield coefficient ($Y_{P/X}$) were calculated according to Eqs. (4) and (5), and (6), respectively.

$$Y_{X/S} = \frac{X_t - X_0}{S_0 - S_t} \quad (4)$$

$$Y_{P/S} = \frac{P_t - P_0}{S_0 - S_t} \quad (5)$$

$$Y_{P/X} = \frac{P_t - P_0}{X_t - X_0} \quad (6)$$

S_t and S_0 are the substrate concentrations at cultivation time t ("t" as index) and at the beginning of the cultivation ("0" as index), respectively. Similarly, P_t and P_0 are the product concentrations at cultivation time t and the beginning of the cultivation, respectively.

The volumetric product formation rate (r_P ; often termed as space-time-yield) was calculated by applying Eq. (7).

$$r_P = \frac{P_t - P_0}{t} \quad (7)$$

2.5 | Determination of the sugar content in the medium

Root cultures were grown in standard medium containing 15 g L^{-1} sucrose. In order to study the necessity of the disaccharide sucrose for the growth, its monosaccharide components glucose (8 g L^{-1}) and fructose (7 g L^{-1}) were used instead as sole carbohydrate sources. Medium (10 mL) was collected every second day during the whole cultivation period, using two biological repeats and four setups in case of shake flask and STBR cultivations, respectively. All media were filtered through a PVDF (polyvinylidene difluoride) membrane syringe filter ($0.2 \mu\text{m}$ pore size; Restek GmbH, Bad Homburg, Germany) and stored at -20°C until further analysis. Sugars were quantified by HPLC (HitachiElite LaChrom, Krefeld, Germany) equipped with a Metacarb column (Varian, Palo Alto, CA, USA), whose sulfonated polystyrene resins in the Calcium form (87C) served as stationary phase and Millipore H_2O as mobile phase. Flow rate was set at 0.6 mL min^{-1} within an oven temperature of 80°C . The retention times of authentic references were approximately 8.91, 10.98, and 13.37 min for sucrose, glucose, and fructose, respectively. Detection was performed using a refractive index (RI) detector. The absolute sugar content was calculated via establishing a calibration curve for each sugar component using its authentic reference.

2.6 | Buffering the medium and measuring the hydrogen ion concentration

After sampling the medium, the hydrogen ion concentration (pH) was measured by the pH meter FiveEasy pH/mV (Mettler-Toledo, Gießen, Germany). The pH of the standard $1/2 \text{ MS}$ medium was set to ~ 5.7 before autoclaving. Two biological repeats were sampled every two days during the whole cultivation period. In order to test the effect of

stable pH on the growth pattern of the adventitious roots, the medium was buffered using citrate (1.71 g L^{-1} citric acid and $1.94 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$) and potassium phosphate ($2.72 \text{ g L}^{-1} \text{ KH}_2\text{PO}_4$ and $0.3 \text{ g L}^{-1} \text{ K}_2\text{HPO}_4$) at pH 4 and 5.7, respectively. Under sterile conditions, the concentrated sterile buffers were added to the autoclaved media to reach a final concentration of 0.02 M for each buffer. Based on the medium composition, the buffer components were selected to avoid the addition of new salts to the medium. Similarly, the pH of the medium was recorded during the cultivation of the root cultures in these buffered media. For STBR cultivation the pH was measured online.

2.7 | Determination of the residual cations and conductivity of the medium

Cations such as Na^+ , NH_4^+ , K^+ , Mg^{2+} , and Ca^{2+} were analyzed as described previously with some modification [31]. The analysis was done via an HPLC system consisting of a Dionex ICS3000 with DionexTM ICS-6000 CD Conductivity Detector (ThermoFisher Scientific, Waltham, MA, USA). Culture media collected at various time intervals were centrifuged at maximum speed for 10 min and filtered through a PVDF membrane. Aliquots ($5 \mu\text{L}$) of $10\times$ diluted samples were injected in an analytical column using a Dionex AS50 autosampler. Separation was performed on a medium hydrophobic Dionex Ionpac CS16 analytical column ($250 \times 3 \text{ mm}$, $6 \mu\text{m}$ 55 % cross-linked ethylvinyl benzene-divinylbenzene grafted with carboxylated functional groups) and protected with a guard column (Dionex Ionpac CG₁₈, $50 \times 2 \text{ mm}$). The chromatographic process was started via isocratic elution of 30 mM methanesulfonic acid at a flow rate of 0.5 mL min^{-1} at 40°C . During the run time (20 min), the retention times of Na^+ , NH_4^+ , K^+ , Mg^{2+} , and Ca^{2+} were 5.5, 6.8, 9.8, 13.0, and 17.8 min, respectively. Data acquisition and the control of the analytical process were done through the Dionex Chromeleon 6.80 software.

The ÄKTA pure instrument (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) was used to measure the variation in the conductivity of the medium during the cultivation time via direct infusion of $300 \mu\text{L}$ filtered medium through the conductivity monitor. A manual flushing of the junctions with 1 mL Millipore water was performed after each measurement. Data were acquired through the UNICORN 6 control software in mS cm^{-1} .

2.8 | Preparation of extracts from the root cultures and determination of their hyperforin content

Lyophilized root biomass was ground with a pestle in a mortar using liquid nitrogen. Ground-dried roots (0.1 g) were placed in a mortar and extracted via continuous grinding for

5 min using 20 mL petroleum ether at 4°C in the dark. The petroleum ether extract was filtered to remove the remaining root material using cellulose filter paper (MN 615 $\frac{1}{4}$, Macherey-Nagel GmbH & Co. KG, Düren, Germany). The filtered extract was then dried under a nitrogen steam in the dark at 4°C . The dried residue was dissolved in 2.5 mL methanol (HPLC grade), which was stored in tightly closed dark glass vials at -20°C after flushing nitrogen gas. When the dried biomass was less than 0.1 g , extraction with mortar and pestle was not efficient. The dried powder was transferred to a 15 mL Falcon tube and extracted with 10 mL petroleum ether via vortexing for 15 min at 2000 min^{-1} (Vortex-Genie, 2 Digital; Scientific Industries, New York, USA). The obtained extract was filtered, dried under a nitrogen steam, and stored as described for large amount of biomass. These extracts were analysed via HPLC to quantify the hyperforin content. HPLC-DAD analysis was carried out as described previously [19], except that the HPLC column used was a XBridge BEH C₁₈ column ($3.5 \mu\text{m}$, $4.6 \times 100 \text{ mm}$). The hyperforin content was determined as described previously [19,20] using authentic hyperforin as a reference to set a calibration curve.

3 | RESULTS AND DISCUSSION

3.1 | The growth characteristics of SJW root cultures

To determine the phase of maximum biomass production, the shake flask-grown root cultures (Figure 2A) were harvested every second day over a cultivation period of 40 days. The stable weight of each freeze-dried biomass (Figure 2C) was estimated. The weights of fresh and freeze-dried biomasses were recorded in order to calculate the moisture content, which showed no appreciable changes during the whole cultivation period when comparing the fresh and dry weights (95.7–99%; Figure 3A). The typical lag phase was observed from 0–14 days of cultivation. The newly transferred roots need this phase to adapt to the fresh medium. The second phase is the linear phase (14–22 days of cultivation), when the maximum rate of increase in the biomass was observed. The last phase in the growth kinetic is the stationary phase (Figure 3A). The growth kinetic during the linear phase was expressed via the volumetric biomass formation rate r_X (Figure 4). The maximum r_X was observed within the linear phase (days 16–22) to be between $6.3\text{--}9.0 \text{ g L}^{-1} \text{ day}^{-1}$ (Figures 3A, 4A). Similar growth patterns were observed for *H. perforatum* and *Echinacea purpurea* root cultures [32,33]. The growth factor g_X was used to define the increase in the biomass at the end of the growth phase (after 40 days). Interestingly, g_X of the shake flask cultivation (40.76, Table 1) was comparable to the values previously reported for STBR cultivations [34]. Hairy root cultures are genetically modified

FIGURE 2 (A) *H. perforatum* auxin-induced root cultures. (B) Adventitious root production in a 3 L-stirred tank bioreactor. (C) Freeze-dried root biomass

FIGURE 3 Time-course study of *H. perforatum* root cultures recording different correlations. (A) Correlation between biomass concentrations and their moisture content as well as pH of the medium. (B) Dependency of the volumetric hyperforin formation rate (r_p) on the carbohydrate content of the medium (sucrose, glucose, and fructose). Data were recorded for the shake flask cultivations in standard medium supplemented with sucrose (15 g L^{-1}) at the beginning of the cultivation period. However, the lower sucrose content at day zero might be explained by poor mixing together with partial hydrolysis of sucrose to glucose (3.37 g L^{-1}) and fructose (1.57 g L^{-1}) during the autoclaving (B)

FIGURE 4 Comparison of the growth parameters of *H. perforatum* shake flask cultivation in standard medium supplemented with either 15 g L⁻¹ sucrose (orange square) or glucose (8 g L⁻¹) and fructose (7 g L⁻¹) (green triangle). (A) Volumetric biomass formation rate (r_X). The green and the orange arrows highlight the shortened lag phase in glucose/fructose-supplemented medium and the prolonged lag phase in sucrose-supplemented medium, respectively. (B) Specific growth rate (μ)

cultures characterized by their fast growth and independence of plant growth regulators. Few reports confirmed the possibility of establishing hairy root cultures from *H. perforatum* explants [21–23,35]. However, none of them tried their cultivation in a bioreactor, nor was growth kinetics recorded at the shake flask level. Depending on the clone and the transformed species, hairy root cultures from other *Hypericum* sp. (*H. tomentosum*, *H. tetrapterum*) showed a 2- to 10-fold increase in the biomass after 22 days of cultivation. The highest g_X was 8.44 for *H. tomentosum*, transformed with *A. rhizogenes* A4 [36]. This value is higher than previously reported data for auxin-induced cultures [37–39]. Impressively, g_X reported in the current study was four to eight times higher than the previously reported values for shake flask cultivations of SJW root cultures (Table 1) and the aforementioned hairy root cultures. Previously, a dramatic variation in the growth of 96 lines of *Gossypium barbadense* hairy roots growing in shake flasks with a g_X value of 426 (0.003–1.28 g

dry weight within 21 days of cultivation) was reported by Triplett et al. [40]. Moreover, a study which derived hairy root-like cultures from *P. ginseng* without *Agrobacterium* transformation confirmed that the season for collecting the explants and the exogenously supplied auxin play an essential role in reproducing a comparable multiplication rate of the newly-induced root cultures [41]. They may help select root lines growing at higher rate than their transgenic hairy root analogues. Thus, fixing these two factors might end in reproducible growth factor g_X through years. Within the same scenario, comparing the growth factors of root cultures developed at the Italian laboratory (Table 1 [37–39]) demonstrated that all cultures possessed similar values even after 8 years of repeated re-establishment of these cultures, which might be explained by the imitated use of the same source of the explants. Correspondingly, the difference in g_X values of the cultures developed in the Korean laboratory was minor when starting with the same inoculum size (Table 1 [29,33]).

TABLE 1 Comparison of the growth parameters obtained from various cultivation trials of *H. perforatum* adventitious root cultures

Type of culture	Capacity of the reactor (L)	Inoculum concentration (g L ⁻¹)	Cultivation		Growth factor g_X^a	References
			Volume (L)	Time (day)		
Shake flask	0.25	5.0^b	0.1	40	40.76^d	Current study
					40^e	Current study
	0.25	2.00 ^b	0.07	14	14.33	[29]
	0.25	0.20 ^c	0.10	23	6.50	[37]
	0.25	5.00 ^c	0.10	23	5.40	[38]
Stirred tank bioreactor (STBR)	0.10	5.00 ^b	0.05	23	~9	[39]
	3.00	5.00^b	1.50	40	28.40^d	Current study
					23.66^e	Current study
	3.00	6.00 ^b	2.00	35	28.31	[29]
	3.00	6.00 ^b	2.00	35	23.46	[33]
	100	3.00 ^b	ND	49	38.00	[34]

ND, not determined. Bold font indicates results from the current study.

^aCalculated according to Eq. (2) at the end of the cultivation period using fresh weights.

^bFresh weight.

^cDry weight.

^dCultures grew in 1/2 MS medium supplemented with 15 g L⁻¹ sucrose.

^eCultures grew in 1/2 MS medium supplemented with glucose and fructose at concentrations of 8 and 7 g L⁻¹, respectively.

3.2 | Changes in the carbohydrate composition affect the biomass production and the hyperforin content

3.2.1 | Root cultures cultivated in sucrose-containing medium

The shake flask cultivations (Figure 2A) were used to record the changes in the composition of the carbohydrates and their consumption during the whole cultivation period. Sucrose consumption in the standard medium, which was supplemented with sucrose, increased in parallel to the increase in the hyperforin concentration, which was proportional to the increase in the biomass (Figure 3A). Roots in the lag phase (day 0–14) had no or trace hyperforin content followed by a gradual increase in r_p from 0.39 to 0.48 mg L⁻¹ day⁻¹ at the start of the stationary phase (day 22–26). The average r_p during the stationary growth phase reached 0.34–0.27 mg L⁻¹ day⁻¹ by the end of the cultivation period (day 38–40; Figure 3B). In the current study, mortar-based extraction method was applied. Previously, Haas et al. [20] had stepwise investigated the efficiency of hyperforin extraction and the downstream processing including the drying parameters. Their results confirmed a maximum hyperforin concentration of 50 mg L⁻¹ after 7 weeks of cultivation when proper downstream processing including the solvent and the extraction devices was selected [19,20]. Very recently, the same hyperforin-rich extract (normalized to 1 μ M hyperforin content) has been proven to have more potent anti-proliferative activity than 10 μ M pure hyperforin [7].

Sucrose was rapidly consumed to be completely abolished at day 10 and remained at the non-detectable level until

the end of the cultivation time (Figure 3B). The decrease in sucrose content was accompanied with a simultaneous increase in the fructose and glucose concentrations starting from 1.57 and 3.37 g L⁻¹, respectively, and reaching maximum concentrations at the beginning of the linear phase (~8 g L⁻¹ each) which yield the sum of the starting sucrose concentration. The linear increase in the biomass concentration paralleled the consumption of the monosaccharides, whose levels diminished gradually until zero by the end of the stationary phase (Figure 3B). The carbohydrate depletion and the consequent start of the root disintegration are suggested to cause the slow decrease of the r_p by the end of the cultivation time.

The continuous growth of roots within the linear phase and the fast hydrolysis of sucrose confirmed that the monosaccharide components rather than sucrose itself are necessary for the biomass production. A similar observation was reported by Cui et al. [33] when complete depletion of sucrose and equivalent formation of its monosaccharides were observed after the first week of cultivation. However, the authors did not test the effect of the exogenously supplemented monosaccharide components on the growth.

Unlike sucrose, whose role as signalling molecule is still to be fully investigated [42], glucose has been recognised as signalling molecule since decades [43,44]. Furthermore, it is important for the expansion of recently divided cells [45]. Prior to its use by the plant cell, sucrose must be degraded by the sucrolytic enzymes invertase or sucrose synthase [46]. In *Arabidopsis*, invertases were shown to be the main sucrose-degrading enzymes, as confirmed by the normal growth of mutants deficient in sucrose synthase isoforms [47].

However, mutation in the cytosolic invertase dramatically affected the growth ending in plants with short primary roots [47,48]. Within the same scenario, the lowest sucrose but highest hexose content correlated with maximum acid invertase activity in carrot roots, which concurred with their fast-growing property [49]. However, a later study showed that the sucrose metabolism in carrot appears to be controlled by both sucrose synthase and vacuolar invertase for utilization and storage, respectively [50]. The total carbohydrate content was almost stable until the start of the linear growth phase, a phenomenon related to invertases whose product is glucose and fructose rather than sucrose synthase, which catalyses the reversible conversion of sucrose to its uridine diphosphate monosaccharide component. Natively grown plants express both intracellular and extracellular invertases [51–53]. However, under shake flask conditions, acid invertases can be secreted into the medium during the in vitro cultivation of plant cells and root cultures. The secreted invertases catalyse the hydrolysis of sucrose into its monosaccharide components glucose and fructose, which agrees with the increase in the biomass parallel to the consumption of the produced monosaccharides [32,54,55]. Similarly, the linear increase in the biomass during the SJW root culture growth is believed to be due to the effect of sucrolytic extracellular invertase and the consequent processing of the monosaccharide products to form new biomass.

Despite the sufficient amount of generated monosaccharides left, the stationary phase started by day 22 of cultivation (Figure 3), which supported the existence of other growth limiting factors like the shake flask space and the possibly subsequent limitation of oxygen. Comparably, bioreactor cultivation of *Atropa belladonna* hairy root cultures ceased to grow when the air saturation reached 50% [56], while 150% air saturation spiked the specific growth rate and the subsequent atropine formation [57].

3.2.2 | Root cultures cultivated in glucose and fructose containing medium

It was reported that the supplementation of one monosaccharide component (either glucose or fructose) to the culture medium resulted in 11- and 14-fold reduction in the multiplication rate of auxin-induced *P. quinquefolium* and *P. ginseng* root cultures, respectively [41]. Nonetheless, the linear growth phase of SJW cultures supplemented with sucrose started only when enough glucose and fructose were generated in the medium (Figure 3A, B). Thus, it was inspiring to test the growth performance of SJW roots inoculated into medium containing both glucose and fructose from the start of the cultivation time. Impressively, these roots grew faster than those growing in sucrose-containing medium. The fast growth was indicated by the shortening of the lag phase by about 6 days, when compared to the sucrose-supplemented roots

(Figures 4A, 5A). In addition, the r_X values recorded for the hexose-grown cultures were four times superior to the cultures supplemented with sucrose only (days 10–16; Figure 4A). The same was true for the μ values calculated within the same time frame (Figure 4B). Expectedly, the increase in biomass concentration agreed with the gradual use of glucose and fructose, which was diminished by day 28 of cultivation. These results indicate that sucrose is not necessary for the growth and a faster adaptation occurs in glucose/fructose-containing medium. This offers the opportunity to shorten the cultivation process for roots growing in monosaccharides-containing medium to maximum ~26 days. Thus, the time of the cultivation process is reduced by approximately 35%, compared to cultures grown in sucrose-containing standard medium until day 40.

The hyperforin production machinery of these fast growing cultures was $0.82 \text{ mg L}^{-1} \text{ day}^{-1}$ at the end of the linear phase (Figure 5B), which was 67% higher than the hyperforin production upon use of sucrose as a substrate ($0.27 \text{ mg L}^{-1} \text{ day}^{-1}$ in the stationary phase; Figure 3B). Moreover, the replacement of sucrose with its monosaccharide components caused a 3-fold increase in the $Y_{P/X}$ (Table 2). The early depletion of the carbohydrates (after day 28) affected the root viability, which was indicated by the deteriorated root morphology mainly in the center of the biomass and the reduced oxygen consumption. Dead tissue leaked its hyperforin content to the medium causing a slow decrease of r_P values from $0.66 \text{ mg L}^{-1} \text{ day}^{-1}$ at day 30 to $0.3 \text{ mg L}^{-1} \text{ day}^{-1}$ at the end of the cultivation period (Figure 5B). Since hyperforin production is coupled to the continuous root growth, prolonging the linear phase by supplying fresh substrates might support the continuous increase in hyperforin production. STBR cultivations (Figure 2B) together with feeding of glucose and fructose before the end of the linear phase are proposed to increase the biomass and hyperforin production.

3.3 | Responses of the root cultures to changes in pH and residual ammonium

The change in pH during the root culturing started with dropping from pH ~6 to about 4.0 after 6 days of cultivation for sucrose-supplemented medium (Figure 3A) and after 2 days of cultivation for monosaccharides-containing medium. The pH remained stable thereafter before it started to rise again to about pH 5.7 after 38 and 30 days, respectively, until the end of the cultivation time (day 40; Figures 3A, 5A). A similar pH profile was previously reported for pear (*Pyrus communis*) cell suspension cultures, which correlated with the biomass pattern [58]. Interestingly, the reported optimum pH value for plant acid invertases was 4 [51,59], which matches the pH value of the medium at day 6 of cultivation (Figures 3A, 5A). This agrees with the increase in the aforementioned

FIGURE 5 Time-course study of *H. perforatum* root cultures recording various correlations. (A) Correlation between the concentrations of the root biomass and their moisture content as well as the pH of the medium. (B) Dependency of the volumetric hyperforin formation rate (r_p) on the carbohydrate content of the medium (sucrose, glucose, and fructose). Data were recorded from the shake flask cultivations in standard medium supplemented with glucose and fructose (8 and 7 g L⁻¹, respectively) at the beginning of the cultivation period

TABLE 2 Yield coefficients for root cultures cultivated in the current study. Values were determined at maximum hyperforin concentration

Type of bioreactor and cultivation volume	1/2 MS-Medium	Substrate	$Y_{X/S}^a$ (g _X g _S ⁻¹)	$Y_{P/S}^b$ (mg _P g _S ⁻¹)	$Y_{P/X}^c$ (mg _P g _X ⁻¹)
Shake flask (0.1 L)	Unbuffered medium	Sucrose	1.376	0.970	0.745
		Glucose and fructose	0.555	1.329	2.467
	Potassium phosphate-buffered medium (pH 5.7)	Sucrose	0.448	0.899	2.000
		Glucose and fructose	1.440	0.870	0.600
Stirred tank bioreactor (STBR) (1.5 L)	Unbuffered medium	Sucrose	0.494	1.063	2.150
		Glucose and fructose	0.358	1.587	4.427

^aBiomass to substrate yield coefficient $Y_{X/S}$ (Eq. (4)).

^bProduct to substrate yield coefficient $Y_{P/S}$ (Eq. (5)).

^cProduct to biomass yield coefficient $Y_{P/X}$ (Eq. (6)).

invertase-mediated hydrolysis of sucrose to glucose and fructose during the cultivation time. Similarly, a close correlation between the accumulation of hexoses and the activity of the cell-wall bound acid invertase was reported for hairy root and cell suspension cultures of *Symphytum officinale* [60].

The early reduction in the pH might be due to the consumption of ammonium (Supporting Information Figure S1). Among all salts constituting the MS-medium, ammonium nitrate (1.65 g L^{-1}) and potassium nitrate (1.90 g L^{-1}) are the major components of the macro-nutrients (full strength MS). Opposite to the low and slow potassium consumption, which was indicated by $\sim 70\%$ residual potassium in the medium (data not shown), only basal ammonium ($\sim 21\%$ equivalent to 178 mg L^{-1}) was detected during the stationary growth phase (days 28–40; Supporting Information Figure S1). Noteworthy, the patterns of Ca^{2+} , Mg^{2+} , and Na^{+} consumption resembled that for K^{+} (data not shown). Ammonium was proven to be essential for the increase in the biomass [61,62]. However, other reports suggested that nitrate is superior over ammonium for the production process of *P. notoginseng* and *H. perforatum* [34,63]. Parallel to the ammonium consumption, values of electrical conductivity were also decreased. Accordingly, the decrease in electrical conductivity was frequently taken as a sign for nutrient consumption (mainly ammonium) and the subsequent biomass increase ([34,58], Supporting Information Figure S1). Similar to the monosaccharide effect, supplementation of ammonium by the end of the linear phase is suggested to delay the stationary phase and to prolong the production machinery.

Since the change in the pH was between 5.7 and 4, the culture medium was buffered to cease its pH change during the cultivation time and to test the effect of the pH on the production process. Cultures cultivated in citrate-buffered medium (pH 4) showed deteriorated root morphology and ceased to grow when compared to roots cultivated in potassium phosphate-buffered medium (Supporting Information Figure S2). Similar to unbuffered medium, sucrose hydrolysis preceded the monosaccharide-dependent biomass production in potassium phosphate-buffered medium (Supporting Information Figure S3A). The $Y_{P/X}$ value of cultures grown in unbuffered medium supplemented with sucrose was 2.7-fold less than the potassium phosphate-buffered counterpart (Table 2), indicating a positive effect of the buffered medium on enhancing the hyperforin production. It was reported that stabilizing the medium pH at 5.5 facilitates the ammonium uptake in vitro [64], thus supporting the observed healthy root appearance and the increased $Y_{P/X}$ of the roots grown in buffered medium (pH 5.7; Table 2, Supporting Information Figure S2). Surprisingly, the use of potassium phosphate buffer failed to stabilize the pH until the end of the cultivation period, which might be due to the low buffer strength (0.02 M). Thus, the increase in $Y_{P/X}$ is suggested to be due to the buffer components rather than the pH itself.

Except for the cultures supplemented with monosaccharides, which exhibited $\sim 25\%$ higher values of $Y_{P/S}$, all shake flask approaches showed comparable $Y_{P/S}$ values independent of the substrate type and the buffering conditions ($\sim 0.9 \text{ mg}_P \text{ g}_S^{-1}$, Table 2). The aforesaid results (Section 3.2.2) indicate that monosaccharides fasten the production process of the root biomass by shortening the lag phase (Figures 4A, 5A), which agreed with their r_P values when compared to sucrose-containing medium (Figures 3B, 5B). These results inspired the possibility of combining the effect of monosaccharides and the buffers in one experiment (Supporting Information Figure S3B). The carbohydrate utilization pattern was comparable to the unbuffered counterpart (Figure 5B). Unexpectedly, this combination did not show appreciable improvement in the r_P value ($0.325 \text{ mg L}^{-1} \text{ day}^{-1}$), when compared to buffered medium supplemented with sucrose and unbuffered medium supplemented with monosaccharides (Supporting Information Figure S3A, Figure 5B). On the contrary, $Y_{X/S}$ was 69% superior over these media, indicating a positive effect of the monosaccharides and buffer combination on the biomass formation without improvement in $Y_{P/S}$ or $Y_{P/X}$ (Table 2).

3.4 | Scale-up potential in bioreactor

Two sets of STBR cultivations (four STBRs each) were processed simultaneously under the same cultivation conditions except for the substrate source (either sucrose or glucose and fructose). Every 10 days of cultivation, one STBR from each set was terminated. Both medium and roots were used to gain insight into the culture performance including the biomass concentration, r_P and sugar concentration over 40 days of cultivation (Figure 6).

The course of the growth shows an almost linear increase in the biomass concentration until the end of the cultivation, which agreed with the reduction in the recorded EC values. The obtained growth factor at the end of the cultivation process was comparable to the previously reported values (Table 1, [29,33]). The pattern of carbohydrate consumption resembled that of flask cultures (Figure 6). Similar to the flask-grown cultures, monosaccharides were superior over sucrose in improving the production yield in STBR by 33% increase in $Y_{P/S}$ and $\sim 50\%$ increase in $Y_{P/X}$ (Table 2). However, there was no significant effect on $Y_{X/S}$. Comparing the r_P values of STBR cultivations to shake flask cultivations gleans a successful cultivation potential with improved r_P of $\sim 0.44 \text{ mg hyperforin L}^{-1} \text{ day}^{-1}$ in STBR supplemented with sucrose versus $0.27 \text{ mg hyperforin L}^{-1} \text{ day}^{-1}$ in shake flask at the end of the cultivation period (Figures 3B, 6A). Nonetheless, the r_P values of the shake flask cultivations supplemented with monosaccharides were 0.83 and $0.66 \text{ mg hyperforin L}^{-1} \text{ day}^{-1}$ after 22 and 30 days of cultivation, respectively, which are 50% and 35% higher than

FIGURE 6 Time-course study of the biomass concentration and the volumetric hyperforin formation rate (r_p) of *H. perforatum* root cultures as well as the changes in the sugar pattern of the medium during cultivation in 3 L-stirred tank bioreactor filled with 1.5 L of medium. (A) 1/2 MS medium supplemented with 15 g L⁻¹ sucrose. (B) 1/2 MS medium supplemented with glucose and fructose at concentrations of 8 and 7 g L⁻¹, respectively. The biomass increase and the corresponding r_p were recorded every 10 days

the r_p values of cultures cultivated in corresponding medium in STBR (0.44–0.49 mg hyperforin L⁻¹ day⁻¹; Figures 5B, 6B). Thus, the full transfer of the cultivation parameters from the shake flask to the bioreactor is still to be amended.

Died root biomass loses its hyperforin content, which is unstable upon leaking to the medium. Different from the peripheral layers of the nourished root material, the central part of the biomass obtained was dark and damaged. This dead tissue was included in the extraction and the analysis process of STBR-produced biomass which affects the total yield of the produced hyperforin. All previously reported bioreactor-based cultivations of *Hypericum* root cultures avoided the use of stirring impellers to reduce the expected damage to the root tip [27,29,33]. Despite the expected poor gas exchange and nutrient transfer, the STBR used in the current study was modified to have a gentle mixing via an impeller to reduce the shear stress. By time the gentle stirring caused the roots to be packed as interlocked mass (Figure 2B) which interferes with the uniform nutrient distribution and creates a heterogeneous oxygen gradient within the produced biomass ending with dead tissue at the centre. To overcome these

drawbacks, special bioreactor setups were reported like gas phase or mist bioreactors. Simonetti et al. [65] showed the possibility of cultivating SJW root cultures in mist bioreactors for 80 days. However, the authors focused mainly on the production of a special antifungal extract without detailed growth kinetics. Roots grown in these reactors were expected to have the least shear stress and growth limitation. Surprisingly, the increase in the root biomass was only 2-fold in the linear phase after 28 days (180–420 g fresh weight, [65]).

The online monitoring of the temperature showed a stable profile at 23°C during the whole cultivation time, while the level of the DO decreased steadily from 5 days of cultivation onwards to reach 75% at day 25. The gradual increase in the DO afterwards indicated a loss of root viability, which is accompanied by the reduction of oxygen consumption to reach only 5% by day 40. The use of STBRs for the cultivation of the root cultures is uncommon. A comparative cultivation was done for hairy roots from *Daucus carota* in an airlift loop reactor and a rotating drum reactor. The volumetric oxygen transfer coefficient (k_La) was taken as a criterion for the bioreactor hydrodynamics, where higher k_La values of

the STBR and drum reactor were merits for the subsequent growth increase in these bioreactors [66]. Consequently, STBRs turn out as future alternative for the cultivation of *Hypericum* root cultures instead of the commonly used airlift loop, bubble column, and mist bioreactors [33,34,65].

4 | CONCLUDING REMARKS

The successful transfer of the cultivation process of *H. perforatum* root cultures including the improved parameters from shake flask to stirred tank bioreactor was shown. The substrate consumption pattern was similar to that of the shake flask cultivation. The results support a monosaccharide-dependent growth. When cultivating the root cultures in glucose and fructose-supplemented medium, shortening of the lag phase resulted in 35% reduction of the cultivation time and a ~3-fold increase in the hyperforin to biomass yield coefficient ($Y_{P/X}$) when compared to disaccharide-containing medium. Similarly, the replacement of sucrose with its monosaccharide components in STBR cultivation aided a 2-fold increase in $Y_{P/X}$, while a 2.7-fold higher $Y_{P/X}$ was observed upon buffering the sucrose-supplemented medium with potassium phosphate. Supplementation of fresh monosaccharides and ammonium by the end of the linear growth phase may improve the biomass yield coefficient and the hyperforin production machinery with a delayed stationary phase. The current study offers a punch of possible correlations and supports the improvements applicable to up-scaling in bioreactors, thus realizing a key step for commercialization. In addition, a sustainable and economically reproducible production of high-value hyperforin-rich extract can offer a reliable basis for clinical trials and open new frontiers for possible industrialization in the future.

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CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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