Asian J. Exp. Sci., Vol. 27, No. 1, 2013; 19-28

On-line Monitoring Plant Biomass for Large-scale Production of *Thymus Vulgaris*, a Medicinal and Culinary Herb

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Abstract : In the present study the mass scale culture of *Thymus vulgaris* L. was made in a Balloon-type bubble column bioreactor (BTBB). It is suggested that *T. vulgaris* can be used as a model system for the formulation of a mathematical model and will be used for on-line monitoring of biomass. The biomass accumulation was monitored within the reactor through measurements of electrical conductivity as the measure of the ion uptake and refractive index as a measure of the sugar uptake. The correlation of electrical conductivity or refractive index of the medium and the biomass has been formulated in the form of a mathematical model as proposed by Ramakrishnan *et al.* (1999). The biomass in terms of dry weight that accumulated after 25 days in the 5 L reactor (BTBB) was 3.151 gm/L. Destructive and on-line methods of biomass estimation gave similar results. The productivity of thymol, an active ingredient with distinct flavour and aroma in the shoots of *T. vulgaris* L was estimated to be 88.228 µg/L for each run in the bioreactor.

Keywords: *Thymus vulgaris*, On-line monitoring, Bioreactor (BTBB), Electrical conductivity, Refractive index, Mathematical model.

Introduction

Thymus vulgaris is a medicinal herb native to the Mediterranean region. The plant can be cultivated at temperature between 12- 15 degree Celsius with mild moisture by shoot cuttings or from seeds. Hence it can be cultivated in India only in the hilly regions. The plant is known for its aroma present maximally in the leaves, stem and purple flowers. The plant is also used for culinary purposes. The whole herb is used in the treatment of digestive disorders, sore throats, fevers, etc., as reported by Selmi and Sadok (2008). The medicinal properties of the herb are due to the essential oil present in the epidermal oil glands. The active ingredients that are present in the oil are monoterpenes and other derivatives. Reda *et al.* (2005) confirmed that the thymol is the major constituent in the essential oil of *T. vulgaris*. On account of its rich source of thymol, the propagation using tissue culture may be employed for a large scale production of the plant. Regeneration of shoots from meristem tips are often used as initial explants for large-scale micropropagation of *Thymus vulgaris* as suggestred by Vila *et al.*, 2002; Prehn *et al.*, 2003.

Ramakrishnan *et al.* (1999) studied the monitoring biomass in root culture systems. They stated that the ability to predict fresh weight is important since it is proportional to the biomass volume fraction that determines mass transfer and other transport characteristics.

Different bioreactor designs are available providing the optimum environment for effective cell growth as well as secondary metabolite production (Eibl and Eibl, 2008). But Balloon-type bubble column bioreactor (BTBB) is more effective for on-line monitoring of plant biomass. Electrical conductivity measurements have been used by many researchers as an indirect method of biomass estimation in on-line monitoring of plant cultures in bioprocess engineering studies for its accuracy and efficiency (Ryu *et al.*, 1994; Thanh *et al.*, 2005). The biomass accumulation can be monitored within the reactors through measurements of electrical conductivity as the measure of the ion uptake and refractive index as a measure of the sugar uptake.

Materials and Methods

In the present study, the aseptic shoot cultures of *T vulgaris* was grown in MS media with 30g/L sucrose, then culture was placed in the 5 L bioreactor. To estimate the biomass that is accumulating during a bioreactor run, two media parameters viz., electrical conductivity (a measure of ionic strength) and refractive index (a measure of sugar content) were estimated. Later, the mathematical equation was used to estimate the biomass accumulated in the reactor. The correlation of biomass and electrical conductivity as well as refractive index has been shown in the work. In this research endevour, methods of on-line estimation of two critical reactor properties have been addressed, namely dry weight indicative of biosynthetic capacity of the shoot cultures of *T vulgaris* L. and fresh weight indicative of actual physical space occupied by the shoot cultures in reactors which has a direct impact on the mass transfer

requirements in a reactor. The correlation of electrical conductivity or refractive index of the medium with the biomass has been formulated in the form of a mathematical model which will enable us to predict the biomass that would be obtained after a run in a Balloon Type Bubble column bioreactor (BTBB).

Shake flask studies: The optimized tissue density for shoot cultures of *T. vulgaris* L were aseptically cultured in sterile modified MS (Murashige and Skoog, 1962) medium with 30 g/L of sucrose.

Optimizing inoculum density: It was decided by growing cultures of *T vulgaris* in 250 mL tissue culture bottles with 30 mL sterile modified MS medium supplemented with 30 g/L sucrose. The inoculum densities in these experiments were varied from 1-10 g FW/L and the duration of growth period was 30 days (corresponding to the log phase of growth). All the experiments were done in triplicates.

Time course profile using shake flasks: To determine the time course profile using the shake flasks, the bottles were kept on a gyratory shaker and harvested in triplicates at intervals of 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 days. Harvested bottles were subjected to fresh weight, dry weight and entrained liquid volume measurements. The fresh weight was estimated by removing the excess medium from the shoots using a blotting paper while the dry weight was estimated after drying the shoots for 48 hours. The freely drained liquid volume was used to estimate liquid medium properties such as electrical conductivity (Model EQ-667 conductivity meter; Equip-tronics, Mumbai, India), refractive index (Temperature compensated ERMA INC hand refractometer, Tokyo, Japan) and pH (Model-PHAN, Labindia Instruments Pvt Ltd, Navi Mumbai, India). The fresh weight and dry weight were determined on a digital weighing balance (Model-AUW220D, Shimadzu Corporation, Kyoto, Japan).

Balloon type bubble column bioreactor (BTBB): Correlation factor obtained from the shake flask studies were used for on-line monitoring of biomass in a 5 L Balloon-type bubble column bioreactor (BTBB) made of borosilicate glass. A sintered glass located at the base of the reactor body was used for aeration and the air flow was adjusted to 3.0 L/min during the cultivation to allow sufficient mixing. The bioreactor has a sampling port at the bottom that allows for the removal of medium aliquots for on-line monitoring. The lid of the reactor is made to fit tightly into the mouth of the reactor and it has two opening which is fitted with tubing made of silicon. One of them serves as an air vent, while the other opening aids as an inoculation port for fresh medium, antifoam, acid or alkali to adjust pH, etc, hence it is fitted with a bacteria proof filter. The bioreactor unit was sterilized by autoclaving at 121 °C and 15 psi for 30 min. The sterile reactor unit was then mounted in a laminar air flow cabinet. Two liters of sterile modified MS medium supplemented with 30 g/L of sucrose was poured into the bioreactor. It was then inoculated with actively growing aseptic shoot explants of *Thymus vulgaris* L at the optimum density that was standardized during the shake flask studies. The reactor was maintained at a temperature of 26 ± 2 °C with a photoperiod of 12 hrs with photon flux density of 70μmolm^{-2}s^{-1}.

On-line monitoring of biomass in the bioreactor: The day of inoculation or the 0 day sampling was done by aseptically aliquoting 2 mL of medium from the bioreactor containing the explant using the side outlet. The medium conductivity, refractive index and pH were measured by the instruments mentioned above. At intervals of 5 days, 2 mL aliquots were sampled for measurement of electrical conductivity, refractive index and pH. Later these data were used to predict the biomass of the explant in the bioreactor.

Results and Discussion

Shake flask studies

The inoculum density selected was 5g/L showing specific growth rate as 0.313 day^{-1}. Inoculation density has been known to affect the growth performances of plants. At very low densities, plants do not grow normally, because of loss of essential substances by diffusion from the cell into the external medium is in confirmation to the observations of George and Sherrington (1984). At higher densities, growth inhibition of plants is known to occur due to production of toxic metabolites. Hence it is necessary to identify an optimal inoculum density to conduct bioreactor studies based on batch growth experiments conducted at different inoculum densities.

Shake flask studies to identify potential on line monitoring technique

The shake flask studies give valuable insight into various aspect of plant tissue culturing using aseptic conditions. This is so because the shake flask studies are the benchmark against which reactors are compared and strategies formulated to get the best outcome from a large scale bioreactor (McKelvey *et al.,* 1993). A number of parameters that were analyzed during the on-line monitoring of shake flask studies of shoot cultures have been compiled in Fig.1. These parameters include electrical conductivity, refractive index and fresh weight of the biomass. These parameters are significant as they help in correlating as well as monitoring the shoot growth in larger volume reactors. The graphical

representation of the growth (Fig.1) shows a pattern of smooth curve that is referred to as sigmoid growth curve for both fresh and dry weight measurements. A growth curve was established to determine the growth characteristics and growth pattern of the shoot cultures. The 'lag' phase was from day 0 to day 10 during which very little growth was observed. The 'log' phase shows a dramatic increase in shoot growth which occurred during day 10 to day 25. During the 'log' phase of growth, the growth rate was calculated and found to be 1.041 g/day with a generation time of approximately 23.064 hrs per gram of the shoot cultures.

Fresh weight and dry weight correlation

The decrease in electrical conductivity and refractive index in the growth medium is consistent with biomass accumulation in shaking flasks. Electrical conductivity is an indicator of the inorganic components in plant tissue culture medium, while refractive index is a measure of the total sugar content in the liquid medium.

A scatter plot of the biomass accumulation in terms of δFW, (where δ indicates difference in final and initial) versus EC based ion uptake, δ(EC V) and refractive index based sugar uptake, δ(RI V) show linear correlations with FW accumulation (Fig. 2 and 3). For *T. vulgaris* L shoot cultures, the ionic yield was 0.0299 g FW per (mS. mL) of EC V while the carbon yield was 0.0565g FW per (g. mL) of RI V. The ability to predict fresh weight is particularly important since this is proportional to the biomass volume fraction which determines mass transfer and other culture transport characteristics. The observation of the present study is in conformity to the findings of Ramakrishnan *et al.* (1999).

A similar scatter plot of the biomass accumulation in terms of δDW versus EC based ion uptake, δ(EC V) and refractive index based sugar uptake, δ(RI V) shows linear correlations with DW accumulation (Figs. 4 and 5). These correlations are not only plots of biomass accumulation versus measured nutrient concentrations (that is electrical conductivity and refractive index) but are also a measure of mass uptake. A linear correlation between DW accumulation and nutrient uptake implies that the ionic yield and carbon yield are constant. For *T. vulgaris* L shoot cultures, the ionic yield is 0.0025 g DW per (mS. mL) of EC V while the carbon yield is 0.0047g DW per (g. mL) of RI V.

Model implementation

Mathematical models of biological processes are often used for hypothesis testing and process optimization. Physical interpretation of results to obtain

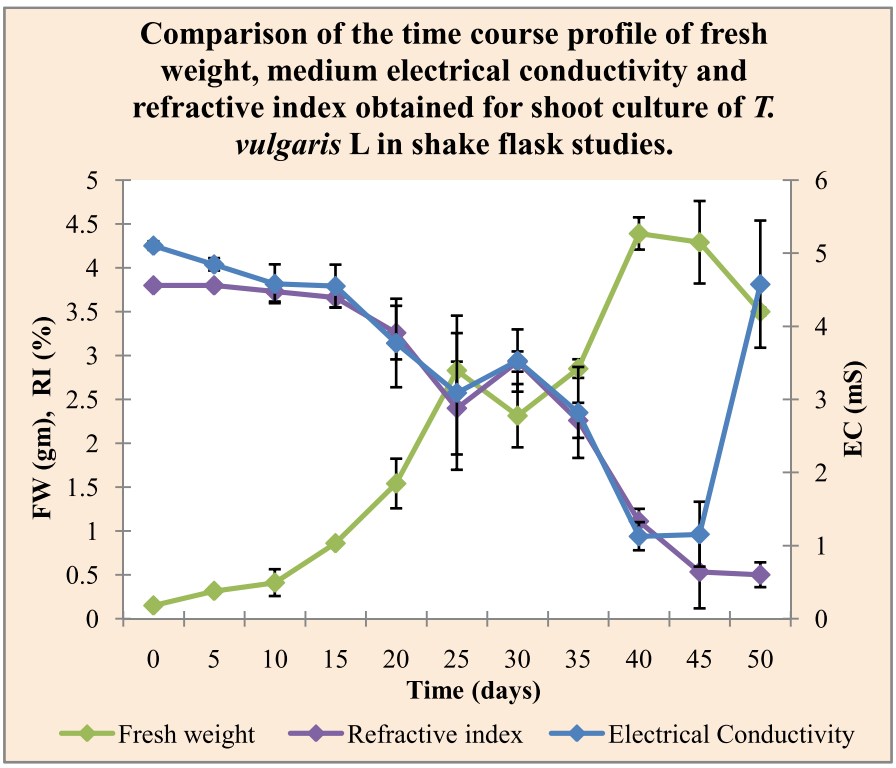

Fig. 1

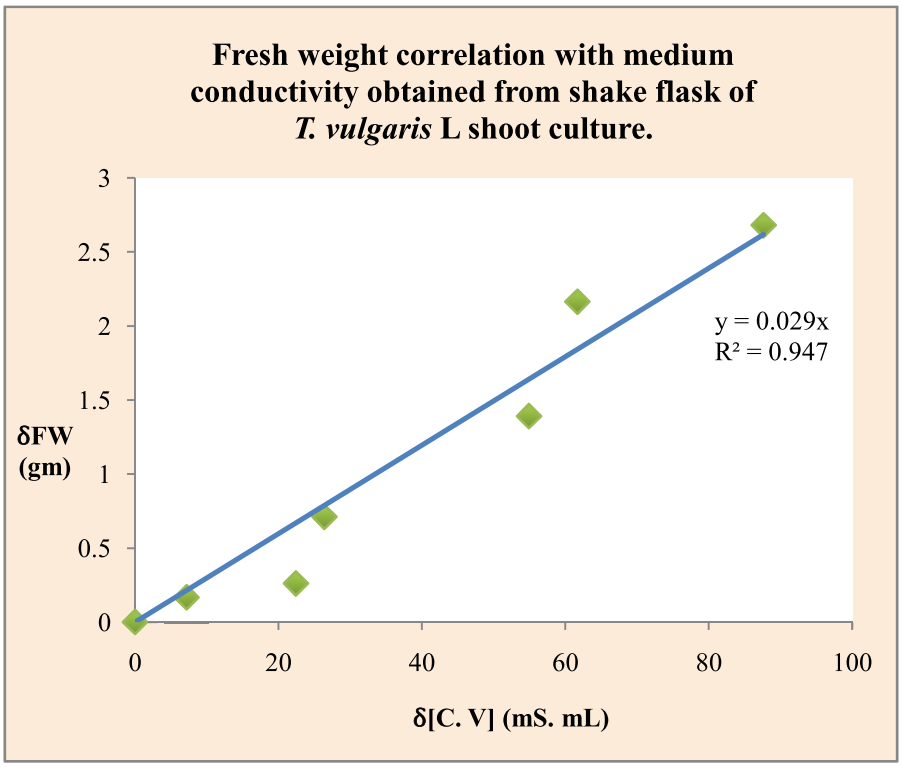
Fresh weight correlation with medium conductivity obtained from shake flask of *T. vulgaris* L shoot culture.
y = 0.029x
R² = 0.947
δFW (gm)
δ[C. V] (mS. mL)
0
0.5
1
1.5
2
2.5
3
0
20
40
60
80
100

Fig. 2

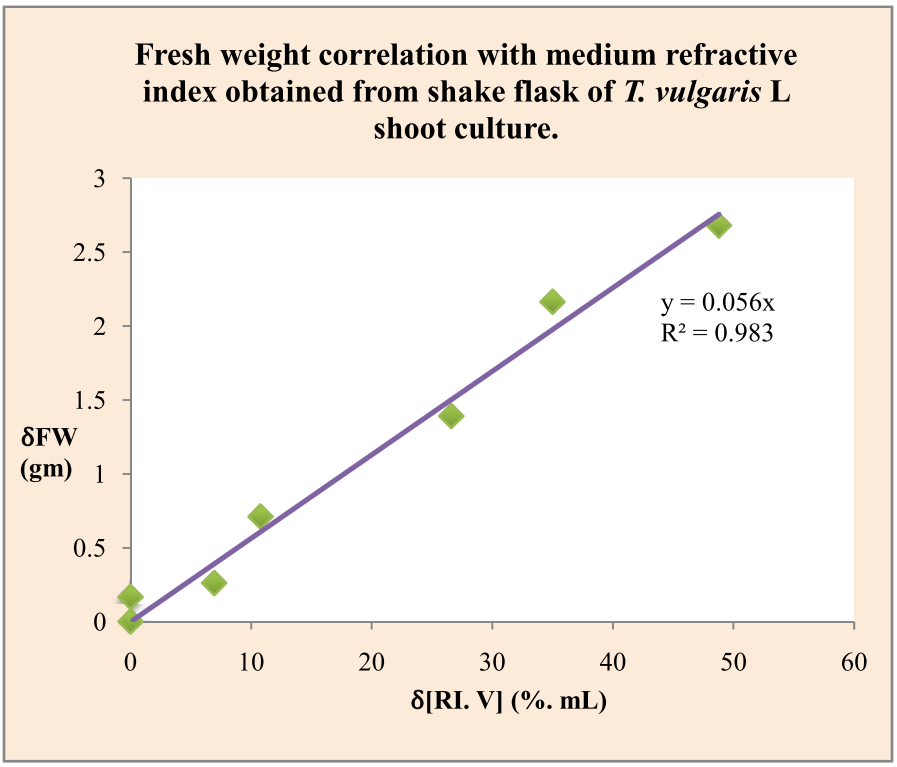
Fresh weight correlation with medium refractive index obtained from shake flask of *T. vulgaris* L shoot culture.
y = 0.056x
R² = 0.983
δFW (gm)
δ[RI. V] (%. mL)
0
0.5
1
1.5
2
2.5
3
0
10
20
30
40
50
60

Fig. 3

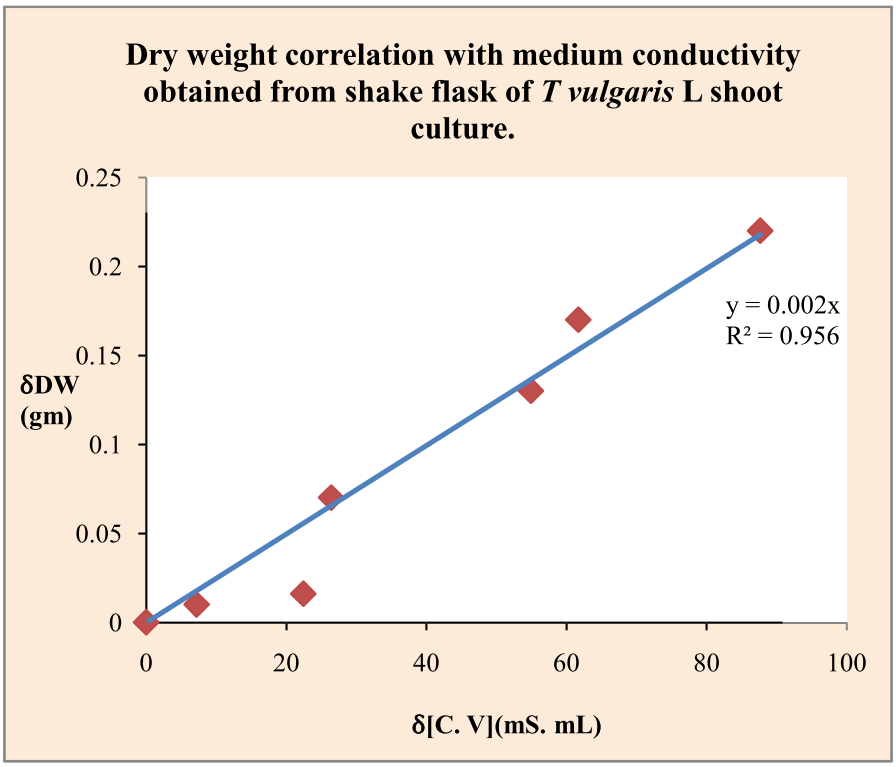
Dry weight correlation with medium conductivity obtained from shake flask of T vulgaris L shoot culture.
0.25
0.2
0.15
0.1
0.05
0
δDW (gm)
y = 0.002x
R² = 0.956
0
20
40
60
80
100
δ[C. V](mS. mL)

Fig. 4

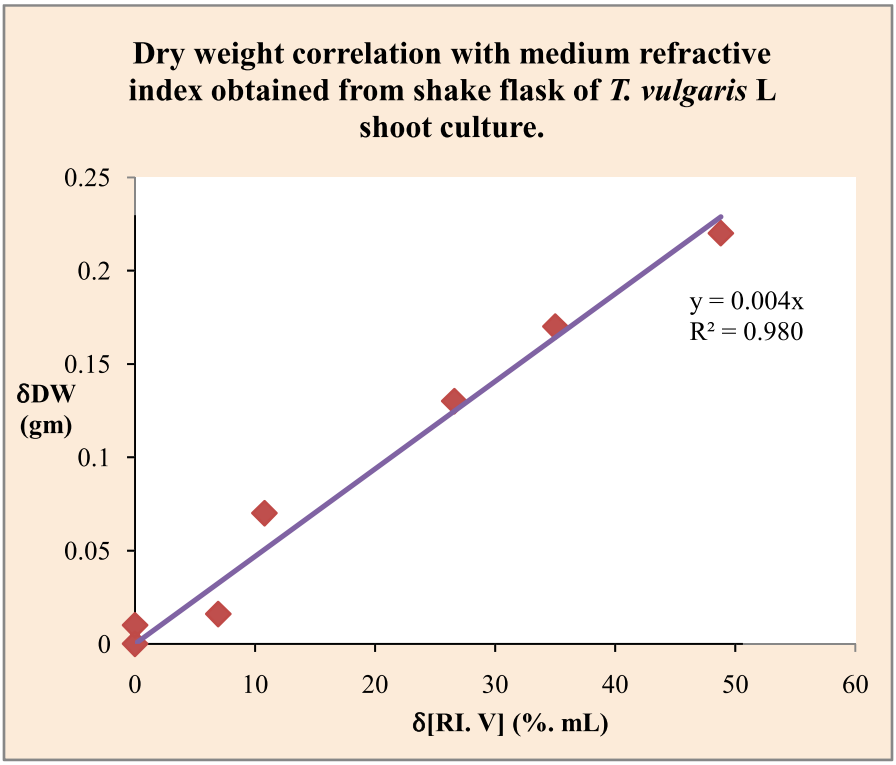
Dry weight correlation with medium refractive index obtained from shake flask of T. vulgaris L shoot culture.
0.25
0.2
0.15
0.1
0.05
0
δDW (gm)
y = 0.004x
R² = 0.980
0
10
20
30
40
50
60
δ[RI. V] (%. mL)

Fig. 5

greater insight into process behavior is possible when structured models that consider several parts of the system separately are employed. The information obtained is very useful for large scale bioreactor cultivation of plant tissues (Ramakrishnan *et al.*, 1999).

Large scale aseptic processing of plant shoot cultures requires on-line monitoring of biomass based on correlation because it is not possible to remove representative aliquots of biomass to verify growth. The reactor growth performance can be expressed as either dry or fresh weight accumulation where the dry weight is the indicator of biosynthetic capacity and the fresh weight is an indicator of physical space occupied by the shoot culture. The typical form of representation of a mathematical model is a simple proportionality between the decline in the medium conductivity and the increase in biomass, which is expressed as under:

$$(DW - DW_{\circ}) = K(C_{\circ} - C) \text{ or } (FW - FW_{\circ}) = K(C_{\circ} - C)$$

(DW = dry weight in grams, $DW_{\circ}$ = initial dry weight in grams, FW = fresh weight in grams, $FW_{\circ}$ = initial fresh weight in grams, C = conductivity in mS, K = proportionality constant (g/mS. mL), $C_{\circ}$ = initial conductivity in mS

A similar correlation based on sugar uptake can also be applied for shoot cultures.

The model was then validated with time course data from the shake flask studies. The empirical correlation was implemented in a mass balance model utilizing conductivity and refractive index. The time course profile of biomass (g DW/L) obtained from shake flask studies for shoot cultures of *T. vulgaris* L grown for 30 days by destructive harvest method and the biomass estimates using conductivity correlation is depicted in Fig. 6. A similar profile was noticed for the refractive index correlation (Fig 7). As is evident from the Figs. 6, 7, 8 and 9, the observed and observed biomass showed increase as a function of time and exhibited similar trend. Therefore, for the scale up studies these empirical correlations were implemented. The proposed model has been validated with time course information (DW and FW) from shake flasks and corroborated with data obtained from the 5 L bioreactors.

On-line monitoring of biomass in BTBB

Fig. 10 depicts the on-line profile of electrical conductivity and refractive index of the medium from the BTBB. The negative slope indicates consumption of ions and sugars by the shoot cultures. Figs. 11 and 12 depicts time course estimates of biomass. Both the graphs show a similar trend in growth.

The growth curve of the shoot cultures failed to show a 'lag' phase which is generally expected, rather the growth pattern is linear and shows only the 'log' phase from day 0 to day 20. The reason for the shoot

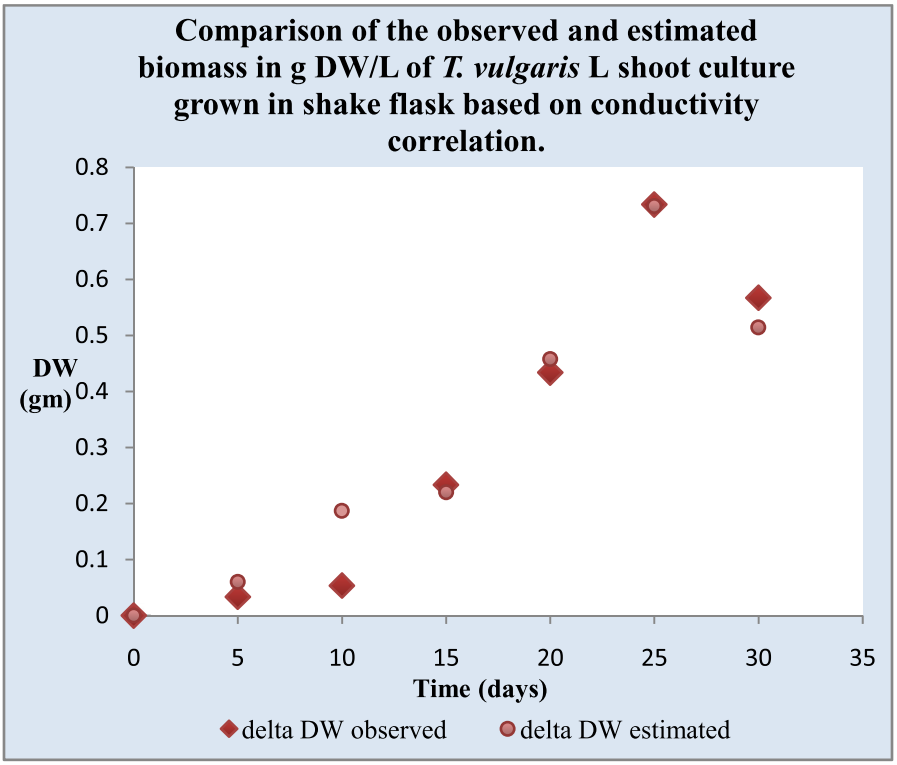

Fig. 6

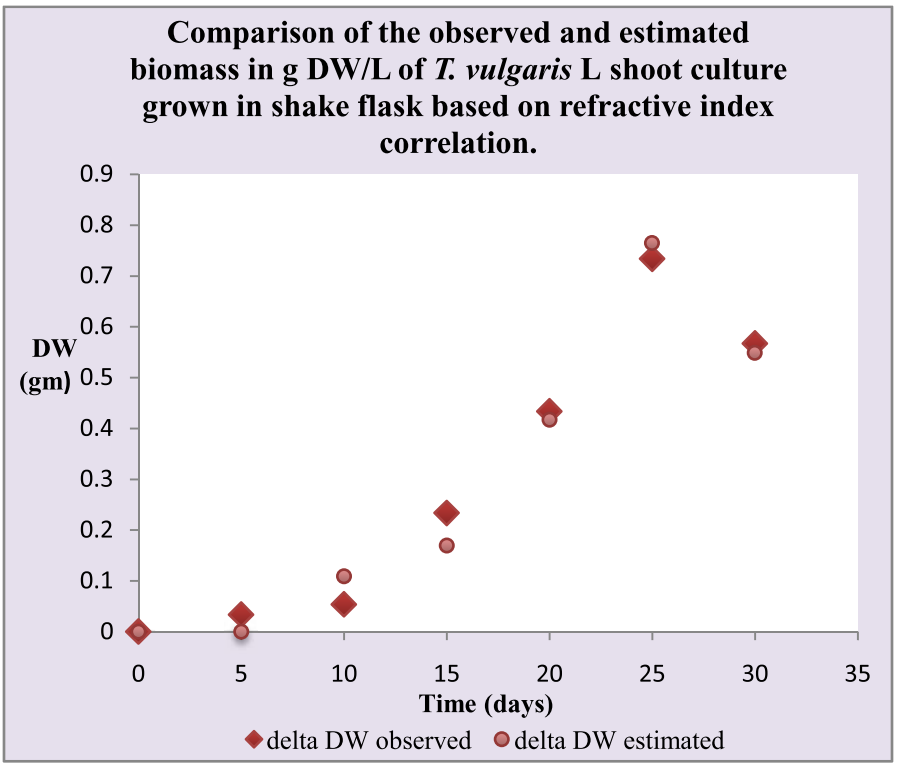
Comparison of the observed and estimated biomass in g DW/L of T. vulgaris L shoot culture grown in shake flask based on refractive index correlation.
DW (gm)
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
5
10
15
20
25
30
35
Time (days)
delta DW observed
delta DW estimated

Fig. 7

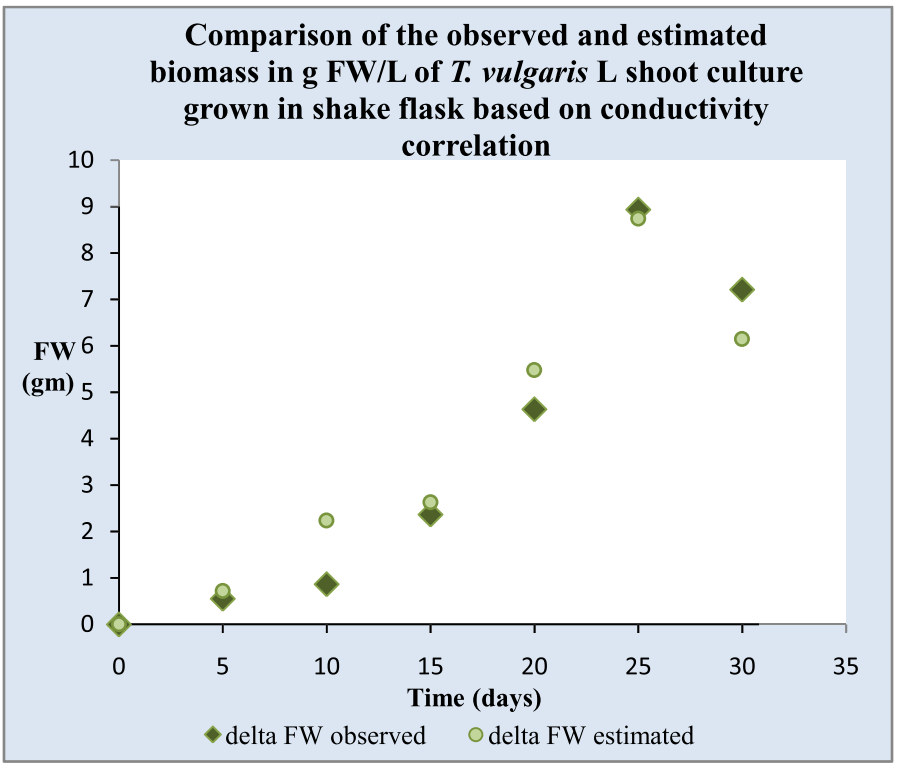
Comparison of the observed and estimated biomass in g FW/L of T. vulgaris L shoot culture grown in shake flask based on conductivity correlation
FW (gm)
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
Time (days)
delta FW observed
delta FW estimated

Fig. 8

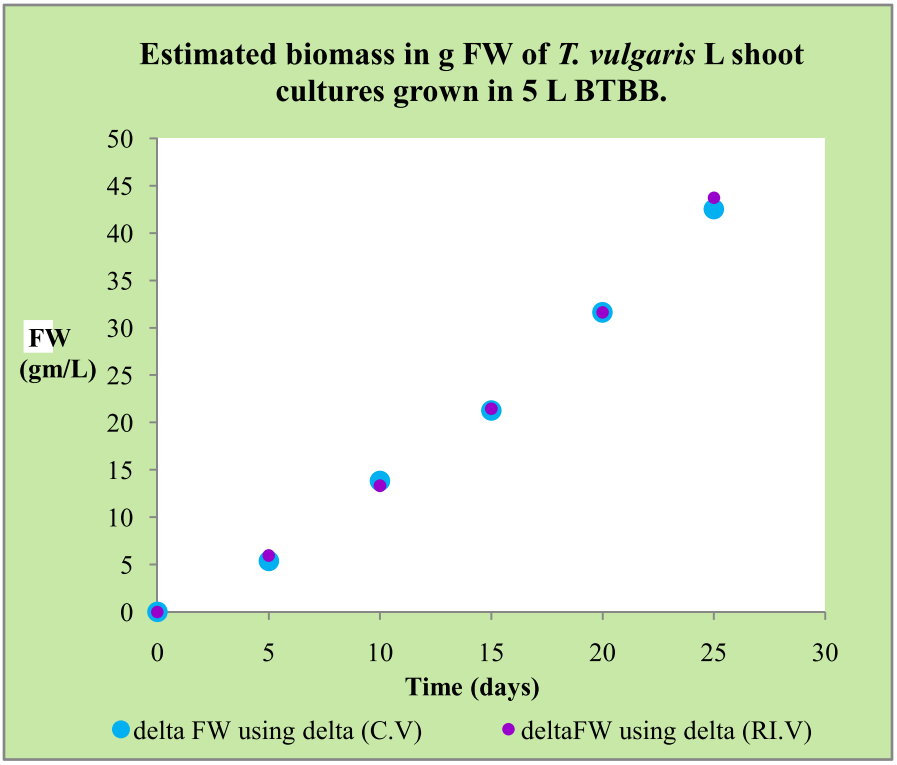
Estimated biomass in g FW of T. vulgaris L shoot cultures grown in 5 L BTBB.
FW (gm/L)
50
45
40
35
30
25
20
15
10
5
0
0
5
10
15
20
25
30
Time (days)
delta FW using delta (C.V)
deltaFW using delta (RI.V)

Fig. 11

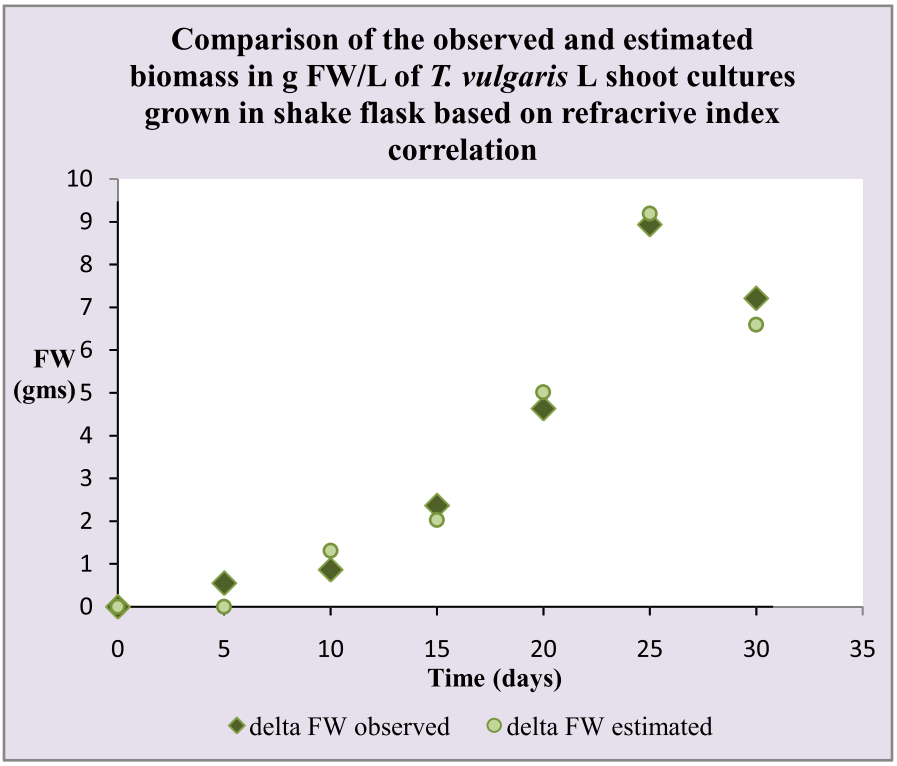
Comparison of the observed and estimated biomass in g FW/L of T. vulgaris L shoot cultures grown in shake flask based on refracrive index correlation
FW (gms)
10
9
8
7
6
5
4
3
2
1
0
0
5
10
15
20
25
30
35
Time (days)
delta FW observed
delta FW estimated

Fig. 9

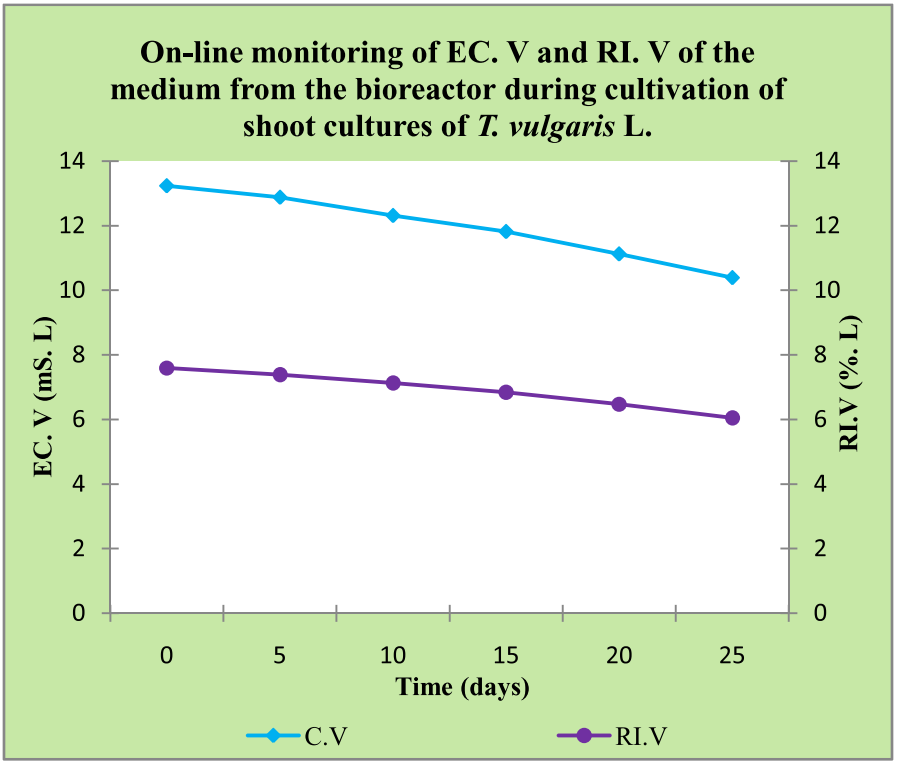
On-line monitoring of EC. V and RI. V of the medium from the bioreactor during cultivation of shoot cultures of T. vulgaris L.
EC. V (mS. L)
RI.V (%. L)
Time (days)
C.V
RI.V

Fig. 10

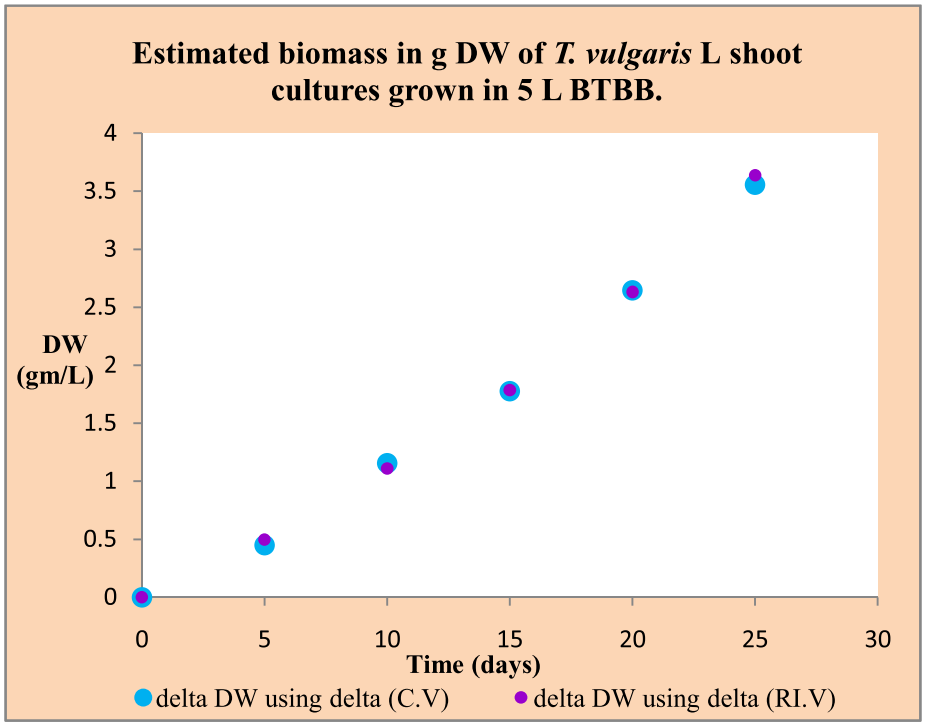
Estimated biomass in g DW of T. vulgaris L shoot cultures grown in 5 L BTBB.
DW (gm/L)
Time (days)
delta DW using delta (C.V)
delta DW using delta (RI.V)

Fig. 12

cultures showing this kind of growth pattern may be due to the stage of growth at which they were seeded into the bioreactor. The shoot cultures were in the 'log' phase of growth in 250 mL tissue culture bottles. They were then transferred into the bioreactor with ample nutrients in the form of ions as well as sugar in the form of sucrose. Moreover, in the bioreactor they are being constantly supplied with sterile air at a constant rate as compared to the shake flasks where aeration is not adequate.

After the 25 day growth of the shoot cultures in the 5 L bioreactor, they were collected and drained off the excess medium. The shoot explants were then weighed and compared to the estimated value. The shoot explants were then dried between sheets of blotting paper at room temperature for 48 hours. The dry weight was also then compared to the estimated values. Table 1 shows the comparison of estimated and actual biomass in terms of fresh weight and dry weight.

The scale-up aspect shows that air driven balloon type bubble column bioreactors are a good option for large scale cultivation of shoot cultures. The refractive index (indicator of sugar content) and electrical conductivity (indicator of ionic content) have been shown to correlate with dry weight as well as fresh weight accumulation of the shoot cultures. The thymol productivity in the shoots of *T. vulgaris* L for each run of the bioreactor was estimated to be 88.228 µg/L.

Table 1 : Comparison of estimated biomass and the actual biomass obtained in the 5 L balloon type bubble column bioreactor.

***T. vulgaris* L shoot culture**	**Observed biomass**	**Estimated biomass**	
		Using EC correlation	**Using RI correlation**
Fresh weight (g/ L)	40.317	42.53	43.731
Dry weight (g/L)	3.151	3.556	3.638

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