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ORIGINAL ARTICLE

In vitro Cultivation of *Jatropha curcas* L. cells for Growth Kinetic and Total Fatty acid Determination

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ABSTRACT
The present study reports, In vitro cultivation of the Jatropha curcas (Linn.) plant cells for evaluation of growth kinetics and total fatty acid content. Plant cells were cultivated in three major synthetic media components in triplicates. Four growth measurement parameters were selected and the data was analyzed using analysis of variance followed by Tukey's honestly significant difference (P< 0.05). Among the parameters; the dry cell and fresh cell weight showed a significant variation between different media components than volumetric measurements. However, a consistent growth kinetics pattern was observed in dry cell weight of cells cultivated within individual medium. The biomass productivity was found to be 4.531, 2.265 and 1.903 gm $l^{-1}day^{-1}$ of cells cultivated in Murashige-Skoog, Woody Plant and Gamborg B5 media respectively. The maximum relative growth capacity of 0.621 and specific growth rate of 0.242 gm day^{-1} was found from cells cultivated in Murashige-Skoog media. The total fatty acid content obtained per dry cell weight was 5 % w/w using acidified petroleum ether extraction followed by transesterification. Thus, the dry cell weight method to determine growth kinetics and total fatty acid content in a liquid system is reliable and reproducible for further bioprocess and biotechnological studies of Jatropha.
Keywords *Biomass productivity. Growth kinetics. Jatropha curcas L. tissue culture*

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INTRODUCTION

The genus *Jatropha curcas* (Linn.) is a tropical and subtropical plant which has multiple potentials and imperative features. Owing to its flexible ecological requirements and easy cultivation practices for various applications; the genus *Jatropha* has taken public attention [1-4]. Although, the plant has defined as a source of biodiesel, cultivation of the plant in a huge mass of land poses irreversible species erosion [5; 6].

Significant variability in planting material, oil content and other important yield components parameters has also been reported as major factors for large scale plantation [6-8]. In addition, favoring certain variety persuades of a single potential use can lead to extinction of the other varieties in which their role in the ecosystem not fully explored. The problems, possible improvement strategies, facts and claims of cultivation of *Jatropha* have been extensively reviewed for the aforementioned purpose and other socio-economic implications [5; 6; 9]. Hence, alternative biotechnological strategies are needed to solve such impediments.

Several researchers have also reported various tissue culture protocols for callus-mediated shoot regeneration from nodal segment, leaf disk, shoot tips and auxiliary buds of *Jatropha* [10-14]. The established protocols either to obtain uniform genotype or disease free planting materials are important but not sufficient to be the main biotechnological tools. The major problems with such protocols are culture viability, delayed cell growth and somatic variation maintenance under *In vitro* conditions.

On the other hand; cultivation of cell suspensions in liquid state have been reported for other species as an alternative system for *In vitro* propagation, *In vitro* selection, germplasm preservation and many transformation systems [15; 16]. Hence somatic variation and totipotency of the culture can be easily maintained by subsequent sub-culturing and various preservation technologies. In addition, it is easy to determine the growth kinetics, the production of metabolites and other physiological activity at a cellular level [17]. Thus, cultivation system in liquid state has remarkable advantages over the conventional solid phase tissue culture and agricultural practice in such regards.

Up to date, there is no or limited report for cultivation of *Jatropha curcas* in a liquid state for any activities. Here we reported *In vitro* cultivation of *Jatropha curcas* in liquid system to determine growth kinetics and the fatty acid content as a method of choice. This work can be helpful for further plant productivity, bioprocess and biotechnological studies to understand and exploit the potential of *J. curcas* for various applications in agriculture and industries.

MATERIALS AND METHODS

Medium composition, culture initiation and maintenance

Establishment of aseptic seedlings and Callus initiation

Seeds of *Jatropha curcas* L. was washed in running tap water for 10 min to remove any adherent particles and kept overnight immersed in distilled water (dH_2O). The husk then carefully removed from the kernel using scissor, gently washed and immersed back in dH_2O containing a few drops of levermed and Tween 20 for 5 min each. Thereafter, the seed was rinsed twice with dH_2O and ready for aseptic operation in a laminar hood. Therein, the seed was surface sterilized in 75% ethanol for 3-5 min and 4% sodium hypochlorite for 18-20 min consecutively followed by three times dH_2O wash to remove all traces of sterilant. The sterilized seeds were transferred to petri dish and blotted on sterile filter paper. The cotyledon leaf disc were excised and inoculated onto the respective growth medium for germination. After 15-days the first emerging leaflets was excised from aseptic seedlings and used as explants for callus induction and the culture was incubated in 16/8hrs photoperiods for one month.

Media composition

Different basal media, MS modification $N_{\underline{0}}$ 3 [18.], Gamborg's B5 [19] and Woody Plant Medium (WPM) [20] supplemented with different plant growth regulators were used for the initiation of callus. In addition to the growth regulators, these basal media were supplemented with 3% sucrose and 0.35% bacteriological agar and 0.12% gelrite (Gelrite® Gelan Gum, sigma). The pH of the media was adjusted to 5.8±0.2 before autoclaving. All chemicals were purchased from Hi Media, India unless and otherwise mentioned.

Suspension culture

Cell suspension culture was initiated from callus cultures grown in a solid agar media for 4weeks. Gamborg's B5, MS and WPM growth media supplemented 2mg/l TDZ, 3mg/1 BAP and 0.5 mg/1 IBA. The culture was then kept in orbital shaker maintained for 120rpm and 16/8 light/dark photoperiods. Henceforth, the stock suspension culture was sub-cultured every10 days to fresh medium in 1:1 ratio. The rest fifty percent of the culture was used for various analyses.

Growth kinetics and fatty acid determination

Cell viability counting

Viability of the cells was checked using Florescen Diacetate (FDA) counter stained with Evans Blue (EB) test every 10 days at the time of sub-culturing. One ml the suspended sample was stained with 10µl of FDA stock and incubated in a complete dark chamber for 15min. The sample then centrifuged 1000 rpm for 10min and the pellet was resuspended in 1ml of dH_2O for 3 min. Then sample was counter stained in 100 µl of 1% Evans blue for 10 min at room temperature [21]. Thereafter, random cell viability count was done by means of a hemacytometer under microscope.

Settled cell volume (SCV) and Packed cell volume (PCV)

Both parameters were chosen for fast estimation of culture growth *In vitro*. Settled cell volume was determined by allowing 10ml of cell suspension to sediment in 15ml graduated conical centrifuge tubes every ½ an hour interval for 4hrs. It was calculated as the percentage of the volume of sediment cell mass to total volume of suspension. On the other hand, the PCV was determined in terms of the volume of packed cell after centrifuging at 1000rpm for 10 min to the total volume of the suspension. Thereafter the culture collected back to respective flasks.

Fresh cell weight (FCW) and Dry cell weight (DCW)

The manipulation of samples was performed in non-sterile conditions taking 2ml of the sample every day for a week and every four days for a month. Fresh cell weight was estimated collecting the cell suspension in a laminar hood using 2ml centrifuge tube and repeatedly washing the cell pack with about same volume of dH_2O. The DCW was determined drying the same cell in the tube at 60°C oven overnight.

Total fatty acid determination

The total lipid was determined using soxlet apparatus in petroleum ether. The sample was first dried in an oven overnight and the dry sample was subjected to extraction apparatus using petroleum ether. The fatty acid content was determined by the change in the weight of the sample in a pre-weighed crucible after extraction and subsequent transesterification per dry cell weight.

Data analysis

The results were analyzed statistically using SPSS vers. 10 (SPSS Inc., Chicago, IL, USA). One-Way Analysis of Variance (ANOVA) followed by Tukey's Honestly Significant Difference (HSD) for mean comparison between values of the treatment was used with 5% level of significance.

RESULTS

Cell Viability and Counting

Florescen Diacetate (FDA) and Evans Blue (EB) stains were selected to monitor the viability during the study time. Random viable cell count was done using hemacytometer under microscope. The subsequent experiments were done if and only if the viable cell count is more than 85 %. The viable cells take up FDA and exclude Evan's blue (Fig .1d).

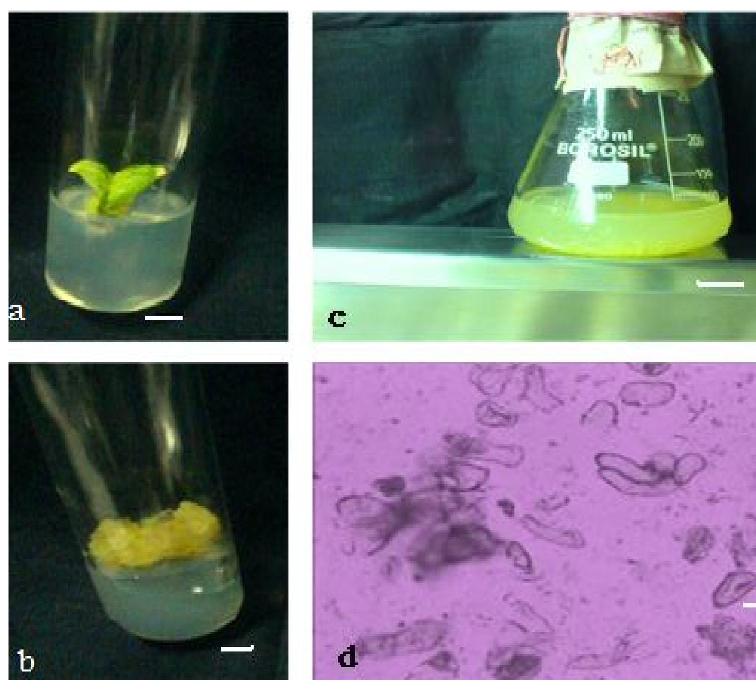

Figure 1. Cultivation of *Jatropha curcas In vitro* in a liquid MS medium: a. two weeks old *In vitro* seedling b. 4 weeks old Callus;c. Suspension culture; d. Viable cells (single or in aggregate). Bars, a and b =1.5cm; c=2cm;d=50µm

Settled Cell Volume (SCV) and Packed Cell Volume (PCV)

Settled cell volume was calculated as the percentage settled cell volume to total volume of suspension. On the other hand, the PCV was determined as SCV after centrifuge the same volume of culture in same tubes at 1000rpm for 10 min. The volume of the settled as well as packed cell was higher in MS medium followed by WPM and B5. Significant difference were observed in SCV and PCV of cells culture with same plant growth media and growth hormone compositions (F=15.63; 19.192; P<0.05). Hence, SCV and PCV were less important to measure the growth kinetics in our study. The test of significance was analyzed using ANOVA and Tukey's HSD (Table 1 and 2).

Table 1. Analysis of variance of the direct measurement parameters in different media components

Parameters	Media	Sum of Squares	df	Mean Square	F	Sig.
FCW (gm)	Between Media	3.96	2	1.982	41.26	.000
	Within Media	2.737	57	0.048		
	Total	6.701	59			
SCV (ml) *	Between Media	21.184	2	10.592	15.631	.000
	Within Media	38.626	57	0.678		
	Total	59.810	59			
PCV (ml) *	Between Media	13.932	2	6.966	19.192	.000
	Within Media	20.690	57	0.363		
	Total	34.622	59			
DCW (gm)	Between Media	0.155	2	0.077	56.212	.000
	Within Media	0.078	57	0.001		
	Total	0.233	59			

* Parametres followed by asterisk showed higher variation within individual media in a replicates (F test =5%)

Table 2. Mean comparison of growth measurements between different media components using Tukey's HSD

Dependent Variable	Media		Mean Difference	Std. Error	Sig.	Confidence interval 95%	
	A	B	A-B			Lower Bound	Upper Bound
FCW	B5	MS	-0.622	0.069	0.000	-0.789	-0.455
		WPM	-0.230	0.069	0.004**	-0.397	-0.064
	MS	B5	0.622*	0.069	0.000	0.455	0.789
		WPM	0.391	0.069	0.000	0.225	0.558
	WPM	B5	0.230	0.069	0.004**	0.064	0.397
		MS	-0.391	0.069	0.000	-.5585	-0.225
SCV	B5	MS	-1.365	0.260	0.000	-1.991	-0.738
		WPM	-0.245	0.260	0.617**	-0.871	0.381
	MS	B5	1.365*	0.260	0.000	0.738	1.991
		WPM	1.120	0.260	0.000	0.493	1.746
	WPM	B5	0.245	0.260	0.617**	-0.381	0.871
		MS	-1.120	0.260	0.000	-1.746	-0.493
PCV	B5	MS	-1.085	0.190	0.000	-1.543	-0.626
		WPM	-0.140	0.190	0.744**	-0.598	0.318
	MS	B5	1.085*	0.190	0.000	0.626	1.543
		WPM	0.945	0.190	0.000	0.486	1.403
	WPM	B5	0.140	0.190	0.744**	-0.318	0.598
		MS	-0.945	0.190	0.000	-1.403	-0.486
DCW	B5	MS	-0.122	0.011	0.000	-0.150	-0.094
		WPM	-0.044	0.011	0.001**	-0.072	-0.016
	MS	B5	0.122*	0.011	0.000	0.094	0.151
		WPM	0.078	0.011	0.000	0.050	0.106
	WPM	B5	0.044	0.011	0.001**	0.016	0.072
		MS	-0.078	0.011	0.000	-0.010	-0.005

* Values followed by asterisk are the maximum biomass mean deviation between MS and B5 media
** Values followed by asterisk shows significant mean variation in PCV and SCV measurements at P< 0.05

Fresh Cell Weight (FCW), Dry Cell Weight (DCW)

The fresh cell weight was measured after the cell mass collected using centrifuge tube followed by subsequent washing and centrifugation. The dry cell weight was measured after drying the same sample at 60°C in an oven overnight. The increase in DCW and FCW of the plant cell in different medium on daily bases for a week and every four days for a month is presented (Fig. 2 a and b). The significance test and comparison of means between various media is shown (Table 1 and 2). From the statistical analysis the DCW was selected among the parameters considered for our study. On the bases of DCW obtained, the Growth Index (GI), Specific Growth Rate (SGR) and Biomass Productivity (BP) were evaluated.

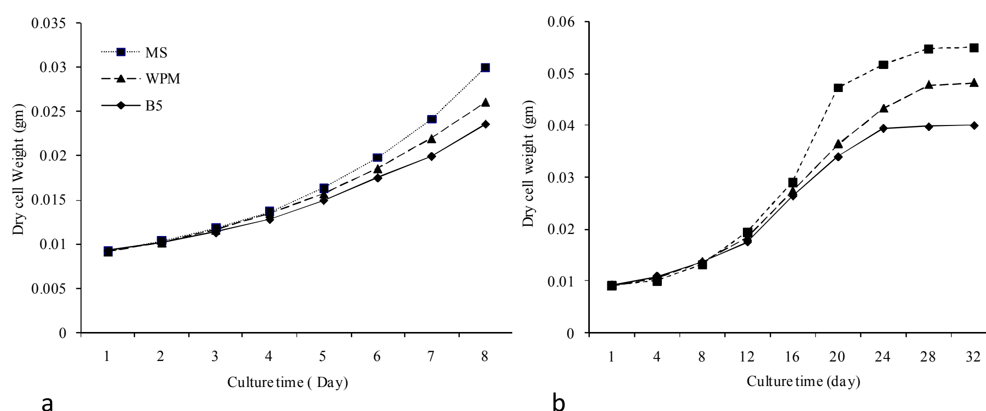

Figure 2. Dry cell mass cultivated in different growth media: a. every day b. every four days

Growth Kinetics: Biomass Productivity, Growth Index and Specific Growth Rate.

Biomass productivity of the cells was determined by ratio of the change in cell weight increase to maximum cell weight per the cultivation time during which the maximum cell weight obtained. The Higher biomass productivity was obtained in MS medium at the 16th day of the culture time. The

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maximum biomass productivity of the cells in B5 and WPM were obtained at 12th day of the culture time. The kinetics of the biomass productivity on the basis of dry cell weight is shown below (Fig. 3).

The ratio of maximum dry cell weight to preceding cell dry weight was expressed as Growth Index. The growth index increases the first 7th days of the culture time in all the three growth media. The maximum increase in growth index was observed at the 16th days of the culture time in MS medium which is higher than the other media components (Fig.4).

The specific growth rate was measured in terms of the biomass gain at specific cultivation time as described (Fig. 5). Like the other growth kinetics measurements the maximum growth rate was obtained at the 16th days of the culture time in MS medium. The culture in MS medium was observed sharply increased especially the first 8 days unlike B5 and WPM. Based on this growth rate, the corresponding doubling time of the plant cells in a liquid system was calculated.

Total fatty acid determination

The total fatty acid was determined using soxhlet apparatus followed by transterification. Dry cell of 1gm in 15ml of petroleum ether was subjected to extraction apparatus followed by transesterification in a reweighed crucible in triplicates. The result is presented as follows (Table 3).

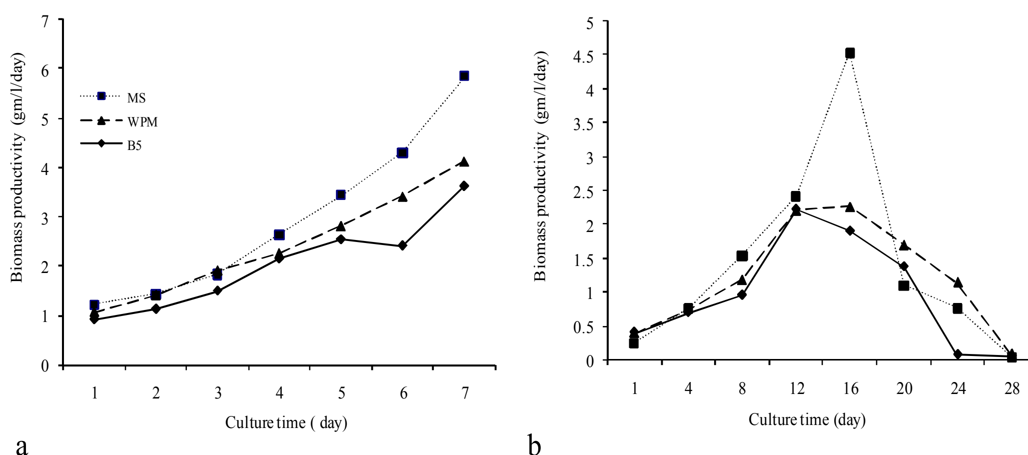

Figure 3. Biomass productivity on the basis of dry cell mass Biomass: data taken a. every day, b. every four days

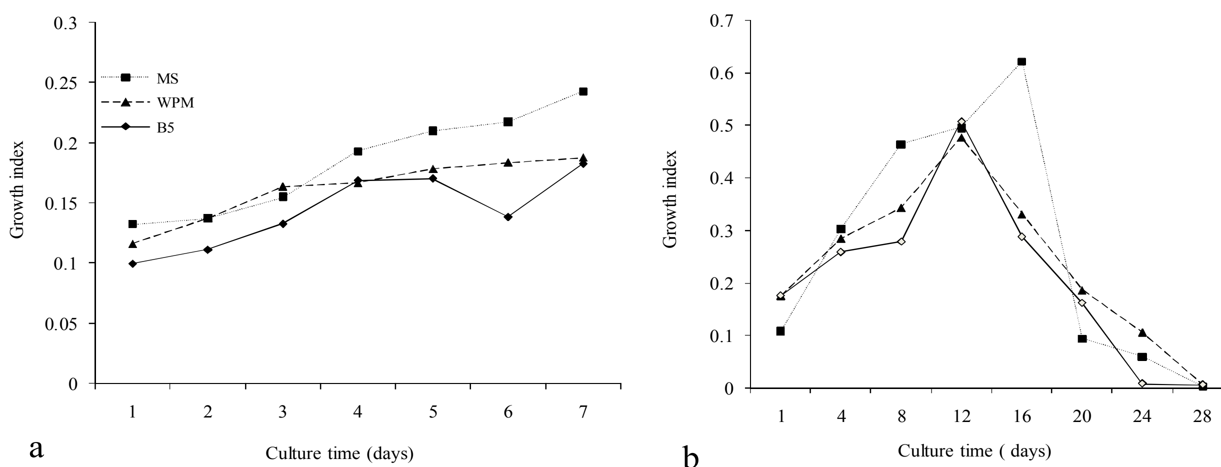

Figure 4. The growth index calculated on the basis of dry cell weight; a. every day; b. every four days

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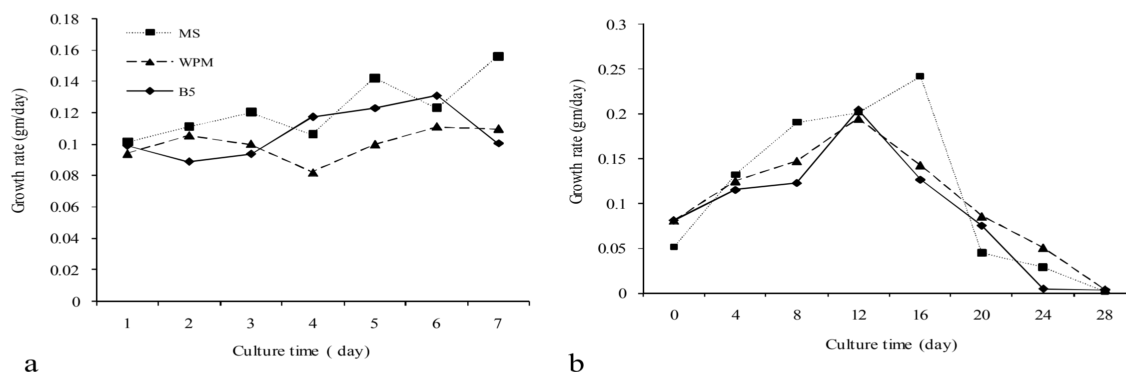

Figure 5. The specific growth rate on the basis of dry cell: a. everyday b. every four days

Table 3. Mean values of fatty acid content of the cells cultivated *In vitro*

Growth Media	Actual wt of the crucible	Wt after transesterification	Difference/1mg dry cell	Percentage (%)
MS	87.11	87.16	0.05	5
WPM	86.69	86.73	0.04	4
B5	85.38	85.42	0.04	4

DISCUSSION

In vitro cultivation of *Jatropha curcas* for growth kinetic and total fatty acid determination was carried out in this study. The growth kinetics is much dependent on physical conditions, type of nutrients and the culturing system. In addition to such physical, chemical and technical elements, reliable and reproducible protocols are vital to determine the plant growth kinetics. The sampling techniques, the type of nutrient, different direct and indirect biomass estimation parameters were found to be the major factors in our study. Various strategies have been reported for the aforementioned purpose elsewhere [22]. However, most of the strategies are indirect and inconsistent.

Biomass estimation in cell suspension cultures of *Capsicum frutescens*, *Pyrus communis* L. and *D. carota* has been described using conductivity, pH and osmolarity of the medium [23; 24]. Unlike the microbial cells, the cell density, the inoculum size and heterogeneous cellular displays of the plant cell are the major impediment for such strategies. For example, the relationship between pH/conductivity change and cell growth has been varied in suspended cells aggregates of *Petroselinum hortense, Glycine max,* and *A. belladonna*; and hairy roots of *S. aviculare* [25].

In our study, packed cell volume (PCV) and settled cell volume (SCV) showed somewhat considerable variation in the growth of cells (Table 1 and 2). The degree of cell aggregates and cell expansion can be the cause for such variation. Moreover, volumetric estimation may be irrelevant to monitor growth kinetic in a heterogeneous pool of cells. Similarly, measurements of PCV and SCV misrepresent cell expansion to a variable extent has been reported [26]. Hence, these parameters were not amenable to measure the growth kinetics in our study. Despite, all these factors both parameters were used for rapid estimation of culture growth under aseptic conditions.

Fresh cell weight (FCW) and dry cell weight (DCW) represent more defined and precise measurements of cell growth than the abovementioned volumetric estimation. Fresh cell weight was rapid for estimation of biomass gain than dry cell weight. However, actual measurement of biomass gain was somewhat variable in FCW across culture period that might be because of water uptake (Table 1 and 2). Both FCW and DCW measurements were performed in non-sterile conditions. Every time; we withdrew 2ml aliquots from the flask in aseptic condition to solve the shortage of the samples in the study time. Hence, during the study time there were sufficient samples for every analysis of the growth kinetics and total fatty acid content.

From our statistical analysis, DCW was selected to determine growth kinetics and total fatty acid content of the suspended cell in different growth medium components. Higher degree of homogeneity within cells cultured in similar medium than between different media components was observed (Table 1 and 2). Hence, the dry cell weight chosen as an essential element to determine the total biomass and more reliably represent cell growth kinetics. The culture time between 8th and 20th days was the period when the cell mass rapidly increased in the dry biomass concentration in all the media components (Fig. 2 and Table 4). This stage might be between the beginning and the end of the exponential phase of the cells in our study.

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Specific biomass productivity of the cells was determined by change in dry cell weight increase from maximum weight per cultivation time. The maximum biomass productivity of 4.531gm $l^{-1}day^{-1}$; which is 4 fold of the previous reports was obtained at the 16th days of the culture time in MS medium (Fig. 3). The maximum productivity of cell cultured in WPM at 16th and B5 at 12th of the culture time was found to be 2.265 gm $l^{-1}day^{-1}$ and 2.223 gm $l^{-1}day^{-1}$ respectively. The maximum biomass productivity of 1.103 gm $l^{-1}day^{-1}$ has been reported from high-density suspension cultivation of *Panax notoginseng* cells in a shake flask [27]. Somewhat equivalent biomass productivity has also been reported in a flask scale high-density batch cultivation of *P. notoginseng* in modified MS medium [27]. This indicates that biomass productivity obtained from our study is worth for production of desirable molecule particularly fatty acid recovery in our study. The biomass productivity was gradually increased from 5th to 28th in WPM media components (Table 4). The importance of biomass productivity determination from small to large scale production of the desire product has been reported extensively [17].

Table 4: Mean growth kinetics measurements of *Jatropha curcas* cultured in major plant growth media components.

Culture time (days)	Biomass productivity ($gm\ l^{-1}day^{-1}$)			Growth Index			Specific Growth Rate ($gm\ day^{-1}$)		
	MS	WPM	B5	MS	WPM	B5	MS	WPM	B5
0	0.25	0.403	0.406	0.108	0.176	0.176	0.051	0.081	0.081
4	0.775	0.765	0.705	0.303	0.284	0.258	0.132	0.125	0.115
8	1.545	1.185	0.955	0.464	0.343	0.278	0.190	0.147	0.123
12	2.417	2.212	2.223	0.496	0.477	0.507	0.201	0.195	0.205
16	4.531*	2.265	1.903	0.621*	0.331	0.288	0.242*	0.143	0.127
20	1.108	1.7	1.378	0.094	0.187	0.162	0.044	0.085	0.075
24	0.773	1.15	0.083	0.059	0.107	0.008	0.029	0.050	0.004
28	0.048	0.09	0.063	0.003	0.007	0.006	0.002	0.004	0.003

* Values followed the asterisk are the maximum Biomass productivity, Growth index and specific growth rate from MS media

The specific growth rate (SGR) and growth index (GI) can show the actual capacity of the growth per cultivation time. The importance of SGR and GI for bioprocess and other scale up studies has been reviewed [17]. In our study, growth index was used to measure association between the relative growth capacities of biomass gain with respect to initial condition at sampling time. On the other hand, the SGR were used to determine the biomass gain of cells culture per unit time. The maximum SGR (0.205 gm day^{-1}) and GI (0.507) were obtained at 12th day and then started decline towards zero around 4 weeks of the culture time in B5 (Fig. 4 and 5). However, these two features were reached to the maximum point at the 16th days in MS (SGR=0.242 gm day^{-1}, GI=0.621) followed by a rapid decrease (Table 4). In conclusion, in the first 8 days of the culture time, GI showed a gradual increase unlike the SGR following a kind of linear fashion in MS medium. From the result it is found that the dry biomass gain were accompanied by the increase SGR and GI and vise versa.

The total fatty acid content was carried out using soxhlet extraction system followed by transesterification. The percent were calculated and found to be 5 % w/w (Table 3). The main objective of this piece of our study was to use tissue culture as an alternative strategy to obtain raw material for biofuel. Because, several researchers have reported the genetic heterogeneity *Jatropha* oil contents and traits [5; 7; 8]. According to these literatures crude oil content ranged from 4-40% and large mass of land is required for mass cultivation of the plant. In addition, the plant through conventional agricultural system bears a seed once in six months or sometimes a year. All these put together indicated the need of new procedures and methods to ensure sustainable raw material for biofuel production. In our study, we showed that importance of continuous cultivation of cells to ensure sustainable production of fatty acid as raw material for biodiesel.

The defatted biomass can also be used as a source of bioethanol after appropriate chemical and enzymatic treatments which we did not include in this part. Among the growth media components used, MS media showed a significant increase in dry biomass. Hence, higher volumes of the dry biomass were used for fatty acid content estimation. There was no that much significant different in total fatty acid content observed among the growth media used a in our study except the difference in dry cell mass (Table 3). The characterization and profiling of the different fatty acids of this plant cultivated in liquid system; however, is not the objective of this study.

In conclusion, reliable and reproducible cultivation of *Jatropha curcas* in liquid state have been carried out. The biomass productivity, specific growth rate, growth index, and total fatty acid content per dry cell weight were determined. The MS medium showed consistent and significant increases in dry cell mass in

general and growth kinetics parameters in particular which was about 2 fold of WPM and B5 at a particular culture time. The fatty acid content obtained even after trasesterification was notable than the crude oil content reported. However, different media components, various biological and physiochemical parameters were the major hurdle to make a strong conclusion about these measurements. It can be considered from the present work that the significant associations of the culture condition and growth kinetics are two vital points in laboratory and/or large scale cultivation of plant cells in liquid system. Hence, optimization of different condition to harvest the desirable plant product is the step for further researches to unify the above dogma. The method established here can be helpful for further studies in regulation of embryonic developments, bioprocesing and biotechnology of *J. curcas* at cellular level for various agricultural/industrial applications.

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